Metabolic and Neurogenic Effects of Amylin in the Rodent *Area postrema*

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“ La struttura alare del calabrone, in relazione al suo peso,

non è adatta al volo, ma lui non lo sa e vola lo stesso”

A. Einstein

1. Summary

Amylin, also known as islet amyloid polypeptide, is co-secreted with insulin by pancreatic β-cells in response to nutrient stimuli. Amylin reduces food intake and body weight and also acts as adiposity signal to control energy expenditure (EE). Circulating amylin acts centrally by primarily activating neurons of the *area postrema* (AP), a circumventricular organ (CVO) located in the hindbrain. Amylin receptors (AMYs) are dimers of the calcitonin-like receptor isoform *a* (CTRa) and of one member of the receptor activity modifying proteins (RAMPs). The presence of CTRa and the RAMPs has been described in several brain areas, including the AP.
Amylin synergistically interacts with the adipokine leptin to reduce food intake and body weight. Although, brain areas where this interaction occurs have not been identified yet, the AP recently emerged as a good candidate. However, evidence of co-expression of all the subunits of the AMYs and leptin receptors (LepRb) in individual AP-neurons is still lacking. Therefore we applied a combination of laser capture microdissection and single-cell qPCR to investigate the co-expression of AMYs components and LepRb in individual AP-neurons. Our results demonstrated that CTRa and one or more RAMPs transcripts are co-expressed in single AP-neurons. Moreover, acute amylin treatment differentially regulates its own receptor: while CTRa is un-affected, RAMP1 and RAMP3 mRNAs are consistently down-regulated. On the contrary, RAMP2 mRNA is up-regulated. Furthermore, 30% of amylin-activated AP-neurons, which co-express all the transcripts necessary to generate a functional AMY, also co-express LepRb mRNA.

Interestingly, these results outline the possibility that more than one RAMP can be co-expressed with CTRa, raising the question whether different CTRa/RAMPs might mediate different physiological effects in the feeding behavior. Indeed, not only does RAMP1 generate an AMY_1 by coupling with CTR, but it also has the potential to bind to the calcitonin-like receptor and generate a calcitonin-gene-related peptide receptor (CGRP). Since both amylin and CGRP decrease food intake and body weight we speculate that RAMP1 might exert a critical role in the regulation of energy balance. In fact, transgenic mice expressing the human (h) RAMP1 (*Nestin/hRAMP1)* on a regular diet are characterized by a markedly lean phenotype. This suggests that RAMP1 might exert a protective effect against obesity. To further validate this

hypothesis, we challenged the transgenic mice with high-fat diet (HFD). Our findings revealed that the lean phenotype is maintained on HFD and the reduction in body weight results from increased energy expenditure rather than from a reduction in food intake. Hence, RAMP1 appears to be involved in the regulation of energy balance.

Finally, CVOs, such as the AP, have emerged as new neurogenic niches in the adult brain, and amylin has been recently shown to exert a neurotrophic effect. Therefore, we combined transcriptome analysis and immunohistochemical techniques to investigated acute and chronic effects of amylin treatment on the AP of adult rats. Our results showed that acute amylin regulates genes involved in multiple pathways and processes of adult neurogenesis. Moreover, chronic amylin infusion increases the number of newly proliferating cells in the adult AP, and determines the fate-commitment of these adult-born cells into mature neurons rather than glia.

In summary, we demonstrated that all the AMY subunits and the LepRb are co-expressed in single AP-neurons. Acute amylin administration differentially regulates the transcripts of the RAMPs and the LepRb in the AP. Additionally, acute amylin affects genes involved in the regulation of multiple pathways and processes of adult neurogenesis. Chronic amylin treatment increases the number of newly proliferating cells in the AP and determines their neuronal fate over glia. Thus, amylin, in addition to its role as a satiating hormone, plays a novel role in the regulation of neurogenic processes in the AP of adult rats.

2. Zusammenfassung

Amylin, auch bekannt als Insel-Amyloid-Polypeptid, wird zusammen mit Insulin nach Nahrungsaufnahme von den pankreatischen ß-Zellen sezerniert. Amylin verringert die Nahrungsaufnahme und die Körpergewichtszunahme bei Labortieren; daneben hat es auch Charakteristika der sog. Adipositas-Signale und ist an der Steuerung der Energieabgabe beteiligt. Das von den ß-Zellen sezernierte Amylin wirkt im zentralen Nervensystem und aktiviert dort v.a. Neurone in der sog. Area postrema (AP), einem Zirkumventrikulär-Organ (CVO), welches sich im caudalen Hirnstamm befindet. Amylin-Rezeptoren (AMY) sind Heterodimere des Calcitonin-Rezeptors, und zwar dessen Isoform a (CTRa), und einem sog. Rezeptoren-Aktivität modifizierenden Protein (RAMP). Amylin interagiert synergistisch mit dem vom Fettgewebe gebildeten Hormon Leptin; eine wichtige Wirkung von Amylin ist, dass es die Leptin-Resistenz bei Übergewicht reduziert. Wo diese Interaktion stattfindet, ist noch nicht genau bekannt; aufgrund früherer Befunde könnte allerdings auch diesbezüglich die AP eine wichtige Rolle spielen. Momentan noch unbekannt ist, ob die verschiedenen Untereinheiten des AMYs und von Leptin-Rezeptoren (LepRb) in einzelnen AP-Neuronen ko-exprimiert sind. Im ersten Teil meines Projekts untersuchte ich deshalb mittels der sog. laser microdissection an AP-Neuronen die Expression dieser Rezeptor-Komponenten auf der Einzelzell-Ebene (single-cell qPCR). Unsere Ergebnisse zeigten, dass CTRa und eines oder sogar mehrere RAMPs Transkripte in einzelnen AP-Neuronen exprimiert waren. Amylin selbst beeinflusste die Expression seines Rezeptors dahingehend, dass die Expression von CTRa unverändert blieb, dagegen war die Expression der RAMP1 und RAMP3 mRNAs nach Amylin-Injektion reduziert. Schliesslich zeigte sich, dass ca. 30% der AP Neurone, die funktionelle AMYs besitzen, ebenfalls die mRNA des LepRb exprimierten. Meine Untersuchungen zeigten folglich, dass in Zellen, die einen funktionellen AMY besitzen, verschiedene Kombinationen von CTRa und RAMP zur Amylin-Funktion beitragen können. Welcher Rezeptor-Subtyp genau für welche Amylin-vermittelte Wirkung verantwortlich ist, ist allerdings noch nicht bekannt.

In weiteren Versuchen untersuchte ich speziell die Rolle von RAMP1 bei der Vermittlung der Amylin-Wirkung; dazu dienten Mäuse, die die humane Version des RAMP1 in Nervenzellen überexprimieren. Die Hypothese war, dass der auf dem

RAMP1-Subtyp basierende AMY v.a. eine wichtige Rolle bei der Regulation der Energieabgabe spielen könnte. In der Tat hatten diese transgenen Nestin/hRAMP1 Mäuse ein niedrigeres Körpergewicht als entsprechende Kontrolltiere, was nahe legt, dass RAMP1 eine schützende Wirkung gegen Adipositas ausüben könnte. Um diese Hypothese zu bestätigen, fütterten wir die transgenen Mäuse auch mit einem Futter mit hohem Fettgehalt. Auch diese Mäuse wogen weniger als Kontrolltiere, was v.a. auf einer erhöhten Energieabgabe als einer verringerten Nahrungsaufnahme zu beruhen schien. Diese Befunde stützen unsere Hypothese, dass RAMP1 bei der Regulation der Energiebilanz eine bedeutende Rolle spielt.

Schließlich zeigten meine Untersuchungen, dass in der AP erwachsener Ratten laufend neue Nervenzellen gebildet werden, d.h. dass es sich bei der AP um eine sog. neurogene Nische zu handeln scheint. Neben einer neurotrophen Wirkung bei neugeborenen Mäusen zeigten unsere Ergebnisse, dass Amylin die Expression von Genen reguliert, die bei verschiedenen Prozesse der Neurogenese beteiligt sind. Eine chronische Amylin Verabreichung erhöhte die Anzahl proliferierender Zellen in der AP, bei denen es sich mehrheitlich um Neurone handelte.

Zusammenfassend zeigten meine Untersuchungen, dass alle AMY-Untereinheiten und auch der LepRb in einzelnen AP-Neuronen exprimiert sind. Erstmals wurde dadurch nachgewiesen, dass auch in nativem Nervengewebe die notwendigen Rezeptor-Komponenten ko-exprimiert sind, und die AP deshalb auch direkt an der Vermittlung der synergistischen Interaktion von Amylin und Leptin beteiligt sein könnte. Weiterhin konnte ich zeigen, Amylin Gene beeinflusst, die an der Neurogenese beteiligt sind. Eine chronische Amylin-Behandlung steigerte die Zahl der neugebildeten Zellen in der AP, wobei es sich v.a. um Neurone handelte. Neben seiner Rolle als Sättigungshormon scheint Amylin deshalb auch eine zuvor unbekannte Rolle bei der Regulation neurogene Prozesse in der AP von erwachsenen Ratten zu spielen.

3. Introduction

3.1. The control of food intake and body weight

3.1.1 Energy balance

Homeostasis (from Greek "*homeo"; similar* and *"stasis"; stable*) is defined as the property of a system in which variables are regulated so that internal conditions remain relatively stable. Homeostasis, strictly applied to humans, could be translated as *"Equilibrium"*. The maintenance of a personal and healthy equilibrium is an everyday challenge. Humans, as with all the other living organisms, depend on energy to perform their normal physiological functions and thus to preserve their homeostasis. Energy derived from food and the body has fine regulatory mechanisms that allow energy absorption, storage and expenditure. To maintain the equilibrium the energy acquired from food and beverages intake needs to be balanced by the energy expenditure (*i.e;* basal metabolism, thermogenesis and physical activity; Figure 1).

How the body (and the brain) achieves and maintains energy balance is not completely understood. A complex physiological network is involved. This system includes peripheral signals regarding energy stores and signals originating from the central nervous system (CNS) that affect energy intake and expenditure (Sandoval et al., 2008). Further, all these signals can influence each other and resulting in a positive or negative energy balance (Jebb et al., 1996; Leibel et al., 1995).

The weight of an individual, at least in adulthood, is one of the most stable parameters and it is consistent with the physiological control of energy homeostasis. A deep understanding of the balance between calories *in* and energy *out* would therefore be fundamental to reduce, and potentially prevent, obesity.

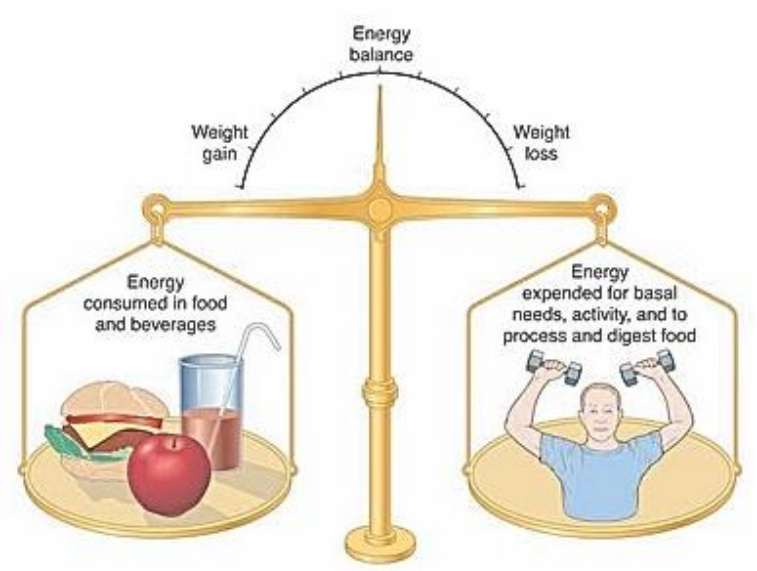


Figure 1: Energy Balance

Illustration describing the maintenance of energy balance. The amount of energy intake (food, beverages) and the amount of energy expenditure (basal metabolism, physical exercises) requires tightly regulated control mechanisms. *http://www.goodtobeyou.nl/calories/.*

3.1.2 Obesity

Obesity is defined as abnormal or excessive fat accumulation that involves risks for the health status. A crude measurement of obesity is the body mass index (BMI) which represents the weight of a person (in Kg) divided by the square of the height (in meters). According to the World Health Organization (WHO) a BMI above 25 kg/m^2 defines a person as overweight, while BMI > 30 represents obesity. The worldwide prevalence of the disorder in adults more than doubled between 1980 and 2014 and currently almost 40% of the population is affected by obesity (http://www.who.int/topics/obesity/en/; Figure 2). Of particular concern is the associated epidemic of obesity in children and adolescents; up to 2013, forty-two million children under 5 years of age were overweight or obese and there is strong evidence of persistence into adulthood (WHO, 2014). Overweight and obesity are associated with substantial increases in morbidity, premature mortality, impaired quality of life and large healthcare costs (Heo et al., 2003; Kopelman, 2000). The major adverse comorbidities include cardiovascular disease, type 2 diabetes (T2D),

metabolic syndrome, gastrointestinal and respiratory difficulties and certain cancers (Haslam and James, 2005). Moreover, adiposity and T2D are significantly linked to an increased risk of developing the Alzheimer's disease (AD) (Arvanitakis et al., 2004; de la Monte and Tong, 2014).

Indeed, obesity is associated with 1 out of 5 deaths among Americans and the illness-related economic costs are approximately $150 billion *per annum* (Finkelstein et al., 2009). Although education of the population to prevent the onset of overweight and obesity is a long-term goal, concrete treatment is actively required for those who are already obese. Surprisingly, successful treatment options still remain quite limited. Changes in lifestyle targeting the everyday diet and the incentive to perform exercise *per se,* unfortunately, do not generally result in marked or sustainable weight loss (Dansinger et al., 2005; Phelan et al., 2006), whereas effective psychological therapies cannot easily be delivered on a mass scale and long-term results are disappointing. Bariatric surgery, such as *Roux-en-Y* bypass or gastric sleeve, is nowadays the most effective treatment in terms of weight loss, comorbidity reduction and enhanced survival (Kral and Naslund, 2007). However, the possible surgical complications and the overall cost make these bariatric procedures strictly reserved for the morbidly obese (Melnikova and Wages, 2006). Hence, obesity still represents a major public health problem. Despite the concern regarding individual lifestyles, also public health and therefore economics are also affected by the prevalence of obesity. Developing effective and long-lasting therapies against obesity appear to be of primary importance. To be successful in this fight, a deep understanding of the mechanisms that control food intake and energy balance and how they are changed in obese individuals is essential.

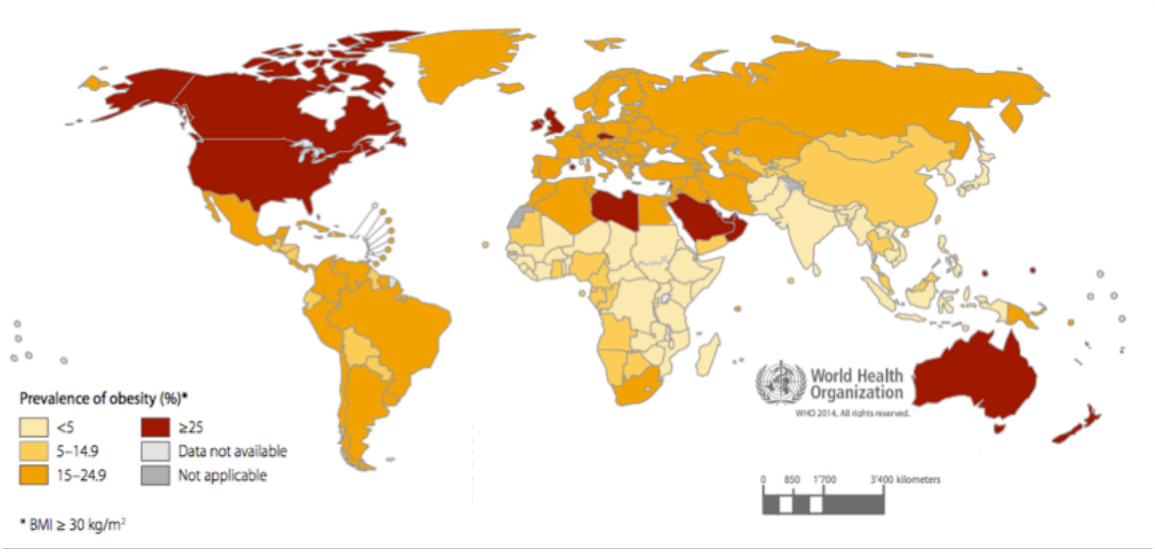


Figure 2: Prevalence of obesity (BMI ≥ 30 kg/m^2) in male adults, 2014

World map showing the prevalence of obesity in male aged 18 years old and over. In 2014, more than half a billion adults worldwide has been classified as obese. Red countries, such as the USA, the UK, Canada and Australia, have more than 25% of obese individuals. Adapted from *WHO, 2014.*

3.1.3 How obesity affects the brain

While the consequences of obesity on metabolic and cardiovascular physiology are well settled, epidemiological and experimental data are beginning to establish that the CNS may also be detrimentally affected by obesity and obesity-induced metabolic dysfunction.

Obesity accelerates the onset of age-related disorders, such as myocardial dysfunction (Hubert et al., 1983), hypertension (Stamler et al., 1978) and stroke (Walker et al., 1996). Therefore, there is a consistent possibility that obesity could also affect aging *per se* and age-related diseases. In the last decade computed tomography and magnetic resonance imaging studies in humans reported an altered morphology in the brains of obese subjects; in particular, a reduction in the whole brain volume was found to be strictly associated with body adiposity (Bruce-Keller et al., 2009; Ward et al., 2005). Further evidence showed that in the brain of obese patients the volume of the focal grey matter is reduced (reflecting a neuronal loss), while the volume of white matter is enlarged (Pannacciulli et al., 2006). Interestingly it has been reported

that such structural changes in the brains of obese patients may partially revert with dieting (Haltia et al., 2007). Moreover, the degeneration of neuronal pathways involved in the onset of neurodegenerative diseases such as AD and Parkinson's disease (PD) may involve compounds (*i.e.,* neuro-peptides) with the ability to cross the blood-brain barrier (BBB) that might exert neuro-toxic actions on neurons. Hormones like amylin and leptin, which are involved in the control of energy homeostasis mainly by reducing food intake and body weight, have the ability to cross the BBB (Banks et al., 1995) and might affect the survival of neurons (Doherty, 2011; Jackson et al., 2013). Reductions in amylin and leptin signaling lead to body weight gain and recent studies also report that low plasma leptin levels are associated with the onset of AD (Lieb et al., 2009). Plasma amylin levels are also reduced in subjects showing signs of mild cognitive impairment and AD (Adler et al., 2014). Furthermore, leptin is able to regulate the secretion and uptake of amyloid beta peptides *in vitro* (Fewlass et al., 2004; Greco et al., 2009). Chronic treatment with the amylin analogue pramlintide increases synaptic protein expression and cognition in a mouse model of AD (Adler et al., 2014). A dysregulation of leptin, amylin and also of the hunger-hormone ghrelin might contribute to the advance of metabolic syndromes, which are correlated with an increased risk of developing cognitive dysfunction and neurodegeneration (Kim and Feldman, 2015; van Dijk et al., 2015). However, those hormones and their mimetic compounds have the ability to inhibit apoptotic and inflammatory processes and to promote cognitive functions, thus resulting in neuro-protective responses in the CNS (Folch et al., 2013). Improving the understanding on the mechanisms by which obesity affects the brain would notably advance the public health and would also help delineate the link between aging, obesity and cognitive decline.

3.2 The peptide hormone amylin

3.2.1 Amylin synthesis and secretion

Amylin, also known as "*islet amyloid polypeptide (IAPP)",* (hereafter referred to as amylin) is a pancreatic peptide hormone. It is co-secreted with insulin by the pancreatic β-cells in a molar ratio of approximately 1:100 (amylin:insulin), in response to nutrient ingestion (Butler et al., 1990). Amylin plays a role in glycemic

regulation by slowing gastric emptying (Young et al., 1995) and promoting satiation (Lutz, 2010a), thereby preventing post-prandial spikes in blood glucose levels. Amylin is processed from the precursor pro-islet amyloid polypeptide (pro-IAPP, pro-amylin, pro-islet protein), which is produced in the β-cells and subsequently undergoes post-translational modifications including protease cleavage to produce the mature peptide amylin (Sanke et al., 1988).

Amylin shares the regulatory promoter motif with insulin and is be influenced by tumor necrosis factor alpha and fatty acids (Cai et al., 2011; Wiersinga et al., 1991). Amylin synthesis is also regulated by factors similar to those influencing insulin synthesis (*i.e;* nutrients, incretin hormones, or neural input) (Butler et al., 1990; Cooper, 1994; Hoppener et al., 2000). For example, during T2D insulin resistance occurs; thus the body does not respond properly to insulin, and this increases insulin production. Since pro-insulin and pro-amylin are co-secreted, this results in an increase in the production of pro-amylin as well. Following the secretion by the pancreatic islets, amylin enters the circulation and it is finally rapidly cleared in the kidney (Vine et al., 1998). The half-life of the secreted amylin is therefore ~15 minutes (Young et al., 1993). Although little is known about amylin regulation, its connection to insulin indicates that regulatory mechanisms that affect insulin also affect amylin.

Amylin acts in response to meals by slowing the gastric empting (Young et al., 1995) and by inhibiting glucagon secretion (Gedulin et al., 1997). The principal role of amylin is its well-described satiation effect, which is achieved by a decrease in food intake mainly by reducing the meal size (Lutz et al., 1995). The area postrema (AP) seems to be a central site that mediates all the amylin effects (Potes and Lutz, 2010; Riediger et al., 2001). Because of all the aforementioned actions, amylin plays a necessary and complementary role to insulin, by controlling the flux of nutrients and the concentration of postprandial glucose (Rushing et al., 2000b). It is generally believed that only the amylin synthetized in the pancreas is responsible for the anorectic effect in rodents. Nonetheless, amylin is also produced by other tissues (*i.e;* endocrine cells of the gastric mucosa and sensory neurons of the dorsal root ganglia) (Mulder et al., 1995; Mulder et al., 1994). Some evidence exists that amylin might also be synthetized in the brain (Skofitsch et al., 1995). However the role of central-produced amylin is still unknown. Only recently, it has been shown that the amylin precursor is centrally expressed in the lateral hypothalamus (LH). Hypothalamic

amylin seems to directly act on LH-neurons that carry the leptin receptor in a similar manner as the adipokine leptin (Li et al., 2015). Thus, central amylin and leptin synergistically interact to regulate long term hypothalamically-mediated feeding behavior. Whether amylin synthetized in the CNS has the potential to reach the AP and act in concert with the pancreatic amylin to directly stimulate AP-neurons is not known yet.

3.2.2 Physiological effects of Amylin

3.2.2.1 Amylin acts as a satiation signal

Amylin's effect in reducing food intake has been extensively described in rodents and humans (Lutz, 2006; Lutz, 2009; Lutz, 2012). Amylin contributes to meal-ending satiation, mainly by diminishing meal size (Lutz et al., 1995). Moreover, in the long-term, administration of amylin decreases food intake and body weight gain in rats.

Acute intraportal or intraperitoneal (*i.p.*) injections of amylin (1-100 μg/kg) significantly decrease food intake in rodents and dose-dependently reduce meal size, without a compensatory increase in meal frequency (Lutz et al., 1994; Lutz et al., 1995). Moreover, peripheral or central administration of the amylin antagonist AC187 stimulates eating by increasing meal size (Mollet et al., 2004; Reidelberger et al., 2004). The lowest dose of exogenous amylin that produces a significant decrease in food intake yields plasma amylin levels slightly higher than those measured post-prandially (Arnelo et al., 1998). Altogether, these findings suggest that amylin is a physiological satiation signal.

Further, amylin chronically infused at high doses (*i.e;* 50 μg/kg/d) displays a stronger eating inhibitory effect in the first few days of infusion compared to later time points (Lutz et al., 2001), indicating a decrease in amylin sensitivity. However, our recent work showed that when rats are chronically treated with more physiological doses of amylin (5-10 μg/kg/d), the animals' responsiveness to acute amylin injections, and thus the decrease in food intake, is maintained over time (Boyle et al., 2011).

The anorectic effect of peripheral amylin is mediated by a direct humoral action on amylin-sensitive neurons in the AP (Lutz et al., 2001). The primary role of the AP in mediating the anorectic effect of amylin is strongly supported by behavioral studies showing that acute and chronic amylin effects are abolished when the AP and the

adjacent parts of the NTS are surgically removed (Lutz et al., 1998b). Further, local amylin administration into the AP reduces meal size and the administration of the amylin antagonist AC187 has the opposite effect; AC187 also abolishes the eating inhibitory effect of peripheral amylin (Mollet et al., 2004). Finally, lesioning of noradrenergic AP-neurons has been shown to completely block amylin's effect on eating, indicating an important role of these neurons in mediating the anorectic action of amylin (Potes et al., 2010b). Electrophysiological and immunohistochemical studies also delineate a direct influence of amylin on AP-neurons (Potes et al., 2012; Riediger et al., 2001; Riediger et al., 2004). Moreover, the AP appears to mediate the amylin's action to slow gastric emptying, because ablation of the AP but not of vagal afferents blocks this effect (Wickbom et al., 2008; Young et al., 1995).

3.2.2.2 Amylin acts as an adiposity signal

In addition to its action as satiation signal, several studies indicate that amylin, like insulin and leptin, shares the properties of an adiposity signal (Hillebrand and Geary, 2010; Woods, 2004). In fact, basal plasma levels of amylin are generally higher in obese rodent and humans (Pieber et al., 1994), suggesting an association between basal plasma amylin and adiposity. Further, chronic amylin administration displays a clear effect in reducing body weight gain, specifically by reducing fat mass, in both rodents and humans (Arnelo et al., 1996; Mack et al., 2007; Roth et al., 2006). Moreover, recent findings describe the effect of central amylin administration in rats that were body weight-manipulated by food restriction or overfeeding. Despite the food testing conditions, rats centrally injected with amylin show a lower body weight compared to controls (Wielinga et al., 2010). Finally, the amylin knockout mouse model is fatter compared to its wildtype controls (Lutz, 2006). Therefore amylin, like leptin or insulin, appears to be an important signal to maintain a regulated level of body weight throughout life.

3.2.2.3 Amylin's effect on energy expenditure

Energy homeostasis not only depends on control of energy intake but also on control of energy expenditure (EE). Several lines of evidence show that acute and chronic amylin administration increases EE in rodents (Mack et al., 2007; Roth et al., 2006). First evidence of this phenomenon described a role of the amylin agonist salmon calcitonin (sCT) to reduce body weight and body fat mass in fasted rats compared to

their controls (Chelikani et al., 2007; Wielinga et al., 2007). Further, several studies demonstrated that the fat-mass reduction was greater in rats chronically treated with amylin compared to their pair-fed controls. This suggests that amylin is also able to increase EE in addition to reduce food intake (Isaksson et al., 2005; Mack et al., 2007; Roth et al., 2006; Wielinga et al., 2010). Moreover, acute amylin administration directly into the third ventricle or into the AP also increases EE (Osaka et al., 2008; Wielinga et al., 2010). Amylin treatment significantly lowers the respiratory exchange ratio (RER) in rodents compared to control baseline, reflecting a shift toward lipid metabolism. All together these findings suggest that amylin might increase EE *via* an increase in fat oxidation (Rushing et al., 2000a). However, the effect of amylin on the reduction of EE might be secondary to the amylin-induced reduction of adiposity or to a possible raise in the metabolic activity of the lean mass (Roth et al., 2006). The physiological relevance of this amylin effect is not known yet. An open question is also which neuronal mechanism mediates this action.

In this regard, it is important to mention that the sympathetic nervous system plays an important role in the regulation of energy metabolism by a direct control on the brown adipose tissue (BAT). BAT plays a primary role in the dissipation energy as heat, thus contributing to energy expenditure by counteracting weight gain with a thermogenic regulation (Seale and Lazar, 2009). Recent studies demonstrate that amylin has the potential to increase BAT thermogenic activity (Fernandes-Santos et al., 2013; Zhang et al., 2011). Interestingly, double transgenic mice expressing the hRAMP1 (*Nestin/hRAMP1* mice) transgene in the central nervous system show an enhanced metabolic response to amylin resulting in a marked reduction of food intake to body weight gain ratio, and in an increased body temperature and EE (Zhang et al., 2011). Furthermore, the increase in EE is mediated by an augmented sympathetic tone acting directly on BAT (Fernandes-Santos et al., 2013; Zhang et al., 2011). The exact neuronal mechanisms behind amylin's action on EE still need to be elucidated.

3.2.3 Amylin receptors

A functional amylin receptor (AMY) results from a heterodimer of the calcitonin receptor (CTR), which belongs to the family of the cell surface bound seven transmembrane G protein-coupled receptors (GPCRs), with one member of the single transmembrane spanning receptor activity modifying proteins (RAMPs)

(Christopoulos et al., 1999). The rat CTR exists in two different isoforms, CTRa and CTRb, resulting from an alternate splicing of the CTR gene transcript. The exact functional relevance for actions mediated by either the *a* or the *b* isoform is not yet fully understood. *In situ* hybridization studies (Barth et al., 2004) that mapped the localization of CTRa/b and RAMPs suggest that only CTRa is present in the rat AP (Christopoulos et al., 1999; Tilakaratne et al., 2000; Watkins et al., 2014).
Three members of the RAMP family have been identified (McLatchie et al., 1998; Sexton et al., 2001): RAMP1, RAMP2 and RAMP3. They are associated in the endoplasmic reticulum and are co-trafficked to the cell surface to form stable complexes that act as chaperons to form different receptors with selective ligand specificity. The dimerization of RAMP1, RAMP2 and RAMP3 respectively, with CTRa, generates AMY_1, AMY_2 and AMY_3 (Alexander et al., 2013; Bailey et al., 2012) (Figure3).

It has been shown that AMY_1 and AMY_3 are characterized by high affinities for amylin and sCT. However, both receptor types have the potential to bind the calcitonin gene-related peptide (CGRP): AMY_1 has a 30-fold higher affinity for CGRP than AMY_3 (Christopoulos et al., 1999), implying that CGRP is most likely to target AMY_1 rather than AMY_3 (Bailey et al., 2012) This suggests that amylin predominantly binds to AMY_3. While all the RAMPs can generate a functional AMY *in vitro* (Christopoulos et al., 1999; Hay et al., 2004; McLatchie et al., 1998), the ability of AMY_2 to bind amylin strictly depends on the type of transfected cell line and on the CTR isoform (Tilakaratne et al., 2000). In fact, CTRb displays greater capacity to dimerize with RAMP2 to generate a functional AMY_2 than CTRa (Morfis et al., 2008). Because the b isoform of CTR is not present in the AP, it is plausible that, in this brain nucleus, RAMP2 mainly couples with the calcitonin-like receptor (CLR) to form an adrenomedullin-responsive receptor (ADM) over an AMY_2. Therefore, different CTR/RAMPs complexes have distinct pharmacology. Further, different AMYs might mediate different physiological effects of amylin (*e.g,* satiation, slowing of the gastric empting, reduction of glucagon secretion) and discerning which subtype is more relevant to each amylin action is still a challenge.

Transgenic mouse models (*e.g.,* RAMPs knockdown) can be instrumental in addressing such questions. Moreover, selective viral depletion of CTR in specific brain areas leads to obesity in rodents. CTR-ablation in the ventro-medial hypothalamus (VMH) of diet-resistant and diet-induced-obese rats on high-fat diet (HFD) results in increased body weight, impaired leptin signaling and insulin resistance (Dunn-Meynell et al., 2015). Thus, the lack of a fully functional CTR signal leads to obesity. Indeed, the core component of the AMY is fundamental to mediate the anorectic action of amylin.

Nestin/hRAMP1 double transgenic mice express the human (h) RAMP1 transgene in the CNS (brain, trigeminal ganglion, spinal cord and dorsal root ganglion), thus allowing the investigation of the central role of RAMP1. *Nestin/hRAMP1* mice are characterized by a marked lean phenotype throughout life (Zhang et al., 2011). The reduction in body weight results from an increased EE rather than from a decreased feeding (Zhang et al., 2011). This suggests that RAMP1 might play a role in the regulation of energy balance *via* a direct effect on EE. However, this study only refers to animals maintained on chow. Whether the lean phenotype is preserved also when mice are challenged with HFD would shed light on the potential role of RAMP1 to prevent obesity. Finally, while RAMP2 knockdown model is not viable, RAMP1 and/or RAMP3 knockdown are currently available and would be an important tool to investigate the different role of each RAMP *in vivo*.

Amylin binding sites have been described in the central nervous system (CNS) as well in the periphery. Peripheral tissues such as muscle, kidney, lungs, bones, stomach and spleen have the potential to bind amylin (Bhogal et al., 1992). The presence of CTR and RAMPs have been shown in the AP (Christopoulos et al., 1999; Tilakaratne et al., 2000; Watkins et al., 2014) and in many different brain areas; such *subfornical organ* (SFO), *organum vaculosum lamina terminalis* (OVLT), *nucleus accumbens*, locus coeruleus, *dorsal raphe*, *fundus striatum*, *nucleus tractus solitarii* (NTS), parabrachial nucleus (PB), central nucleus of the amygdala, *bed nucleus stria terminalis* and hypothalamic areas: specifically in the *nucleus arcuatus* (Arc), VMH, and dorso-medial hypothalamus (DMH) (Becskei et al., 2004; Christopoulos et al., 1995; Le Foll et al., 2015; Sexton et al., 1994; Skofitsch et al., 1995). Finally, lately, the mRNA for all the components necessary to form a functional AMY have been described in the ventral tegmental area (VTA) (Mietlicki-Baase et al., 2015a). However evidence of

co-expression of all the AMY subunits in the same cell and in native tissue is still missing.

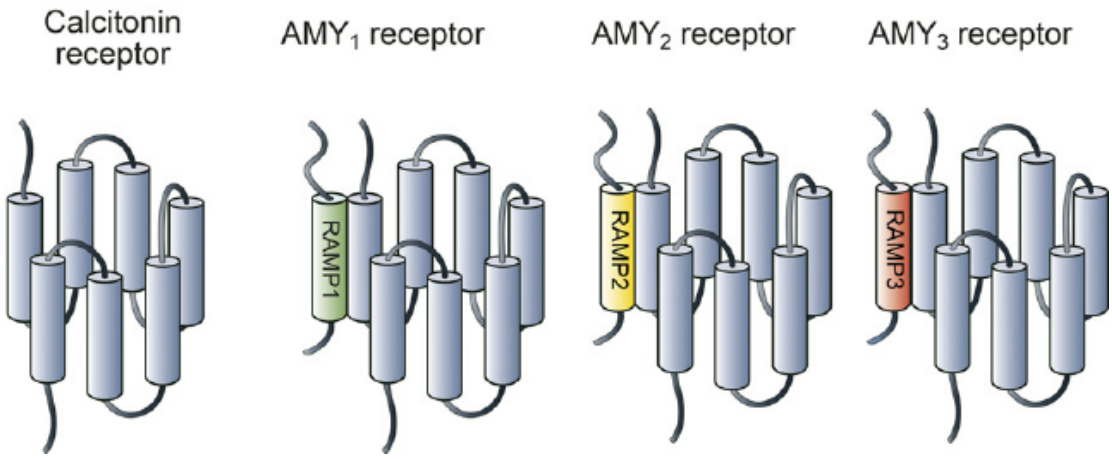


Figure 3: Schematic diagram of the amylin receptors

Amylin receptors are formed by the interaction of the seven transmembrane calcitonin receptor (CTR) with a member of the single transmembrane spanning receptor activity modifying proteins (RAMPs). The heterodimerization of CTR with RAMP1, RAMP2 and RAMP3 generates the AMY_1, AMY_2, AMY_3 receptors, respectively (Hay et al., 2015).

3.2.4 Central amylin signaling

The early gene *c-fos* is a common marker for neuronal activation and its expression is used to define brain areas that might be involved in responses to specific stimuli. Extensive Fos immunohistochemistry demonstrate that the neuroaxis activated by amylin also comprise the NTS and the lateral parabrachial nucleus (LBP) (Becskei et al., 2007; Riediger et al., 2004; Rowland et al., 1997). Neuronal tracing studies with anterograde and retrograde tracers confirm the direct links between these amylin-activated areas. These studies also identify the LPB as the primary relay station between the hindbrain and the hypothalamus, including the lateral hypothalamic area (LHA), where amylin reduces fasting-induced c-Fos expression (Potes et al., 2010a; Riediger et al., 2004), and the VMH where histamine H1 receptors may be implicated in amylin's effect on eating (Mollet et al., 2003).

Moreover, amylin-induced Fos-response in AP-neurons markedly overlap with tyrosine hydroxylase cells; suggesting that the dopaminergic system might also play a role in amylin's inhibitory effect on eating (Potes et al., 2010b). Additionally, amylin time and dose-dependently activates the extracellular signal-regulated kinase 1 and 2 (ERK 1/2) cascade by inducing ERK phosphorylation (Potes et al., 2012). Furthermore, the number of pERK-positive AP-neurons is higher 10-15 minutes after acute amylin administration, which is the time window in which the satiation effect of amylin is at its maximum (Lutz et al., 1995; Potes et al., 2012). The 20% of pERK-positive AP-neurons are characterized as noradrenergic neurons, showing positivity for the dopamine-beta-hydroxylase marker (Potes et al., 2012). Finally, after acute amylin stimulus, pERK neurons in the AP partially overlap with the CTR (Potes et al., 2010b). All these findings support the idea that ERK signaling cascade is employed by amylin to induce meal termination in the AP. Further, immunohistological and electrophysiological evidence indicates that the amylin's excitatory effect on AP-cells is mediated by an increased formation of cGMP (Riediger et al., 2001). Finally, a recent electrophysiological study demonstrates that AMYs are mostly found on presynaptic glutamatergic terminals in the AP-neurons, suggesting that glutamatergic transmission in the AP might also contribute to mediate the amylin's effect (Fukuda et al., 2013). Despite these findings describing a plausible mechanism of amylin's anorectic action, many questions still remain to be answered to obtain the full picture on how amylin signaling is integrated and processed by the brain.

3.2.5 Amylin and leptin interaction

Amylin is also known to synergistically interact with the adipokine leptin to decrease body weight and food intake (Roth et al., 2008; Trevaskis et al., 2010b). Leptin is secreted by adipose tissue (Zhang et al., 1994) and regulates energy homeostasis by reducing food intake and body weight. The leptin pathway involves a number of brain areas, in particular the *Arc*, the VMH and the NTS in the hindbrain. Leptin influences various intracellular signaling cascades in leptin-responsive neurons, including the janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) signaling cascade (Baumann et al., 1996; Ghilardi et al., 1996; Ihle and Kerr, 1995). Physiologically, plasma leptin levels rise in response to fat accumulation in adipose tissue, eliciting an anorexigenic response in leptin sensitive individuals (Mainardi et

al., 2013). On the contrary, when leptin levels are low all actions are disinhibited: food intake increases and energy expenditure decreases.

It is important to realize that physiologically the sensitivity of the leptin signaling system seems to be rather asymmetric. Accordingly, most common forms of obesity are associated with high leptin levels and leptin resistance, and further elevations of leptin have very little effect. Hence, the system seems to be primed in a way that low energy stores lead to a necessary increase in caloric intake (and decrease in energy consumption) but that high-energy stores and hence high leptin levels typically cannot prevent the development of obesity or reduce obesity once it is established. Several studies have shown that the anorexigenic effect of leptin seems to be markedly enhanced by amylin (Roth et al., 2008; Trevaskis et al., 2010b; Turek et al., 2010). Indeed, several lines of evidence demonstrate that combined treatment of amylin and leptin has beneficial effects, the most notably being amylin-induced restoration of leptin responsiveness in obese humans and rats (Roth et al., 2008).

Leptin exerts its effects by binding to the b isoform of the leptin receptor (LepRb), and the VMH might be the site mediating the amylin-leptin interaction (Le Foll et al., 2015; Trevaskis et al., 2010b; Turek et al., 2010). Other brain areas, such as the AP (Roth et al., 2008; Trevaskis et al., 2010b) or the VTA (Mietlicki-Baase et al., 2015a) may also play a role. A recent electrophysiological study demonstrates that 50% of AP-dissociated neurons are excited by both amylin and leptin *via* an increased cAMP intercellular signaling mechanism (Smith et al., 2015). Moreover, previous findings demonstrate that pSTAT3 signaling is elevated in the AP in response to leptin (Huo et al., 2007). These findings suggest that a neuronal population responsive to both amylin and leptin might be present in the AP, however evidence of co-expression of AMY and LepRb in individual AP-neurons is still lacking. Understanding the brain regions where leptin and amylin interact, and how amylin agonism improves leptin sensitivity, would be beneficial to combat leptin resistance and thus to treat obesity.

3.2.6 Clinical relevance of amylin and leptin interaction

Several line of evidence demonstrate that amylin and leptin functionally interact to reduce food intake and body weight gain, suggesting that the combined treatment with amylin and leptin might be helpful to develop anti-obesity drugs. Pair-feeding studies

on lean and obese rodents have shown that pharmacological levels of chronic peripheral amylin administration (300 µg/kg × d) in diet-induced obesity-prone animals is of similar magnitude to that observed in lean animals (Roth et al., 2008). Thus, chronic amylin infusion reduces food intake and body fat mass without any compensatory decrease in EE (Roth et al., 2008). The amylin analogue pramlintide is currently used in the treatment of type 1 and type 2 diabetes mellitus. Pramlintide induces weight loss and decreased eating (Chapman et al., 2005). While amylin-analogue monotherapy is beneficial several studies suggest that the combination of amylin and leptin is a very effective treatment against T2D and obesity (Ravussin et al., 2009). Co-administration of amylin and leptin has been shown to exert a synergistic effect on weight loss. Administration of leptin alone, even at relatively high doses, leads to leptin-resistance (Roth et al., 2008). Amylin and amylin agonists are able to partially restore leptin responsiveness in obese rodents and humans. The synergistic effect results in a fat-specific reduction of weight and this effect might be mediated by an amylin-improved leptin signaling in the hindbrain (Ravussin et al., 2009; Roth et al., 2008). Further studies are still required to define the possible outcomes of a prolonged treatment and to evaluate the consequences at the end of the therapy.

3.3 Adult neurogenesis

3.3.1 Adult neurogenesis in the mammalian brain

Neurogenesis is defined as the process of generating functional neurons from neural stem cells (NSCs) and progenitor cells. It drives nervous system development and is mainly active during embryonic and pre-natal stages (Ming and Song, 2005). However, in restricted brain areas, neurogenesis still occurs in the adult mammalian brain. The first anatomical evidence for the presence of newly generated cells in the adult brain was found in the post-natal rat dentate gyrus (DG) (Altman and Das, 1965). The functional integration of newly generated neurons into the pre-existing circuits in the CNS was first shown in songbirds (Paton and Nottebohm, 1984) and multipotent NSC were later identified in the mammalian brain (Reynolds and Weiss, 1992; Richards et al., 1992)

Active adult neurogenesis is patterned more in space rather than time. In fact, there are two main neurogenic niches in the adult brain: the subgranular zone (SGZ) of the hippocampus (Eriksson et al., 1998; Kempermann and Gage, 2000) and the subventricular zone (SVZ) lining the lateral ventricle (Doetsch et al., 1999). Newborn neurons generated in the SGZ migrate into the granule cell layer of DG, while newborn SVZ-neurons migrate along the rostral migratory stream to reach the olfactory bulb and become interneurons (Figure 4). However, other brain regions might also participate in the generation of new neurons. In fact, adult neurogenesis is a dynamic, finely-tuned process which is subjected to modulation by various physiological, pathological and pharmacological stimuli. Several lines of evidence demonstrate that ischemic injury leads to an increased neurogenesis in the adult mammalian brain and this phenomenon might be a source for self-repair (Lin et al., 2015; Macas et al., 2006).

The NSCs in the adult brain preserve the ability to self-renew both neurons and glial cells (Okano, 2002; Palm et al., 2015). The mechanisms that specify cell fate in adult progenitors are finely regulated by extracellular and intracellular programs (Zhao et al., 2008) which guide the adult-born cells through proliferation, specification and differentiation. When a NSC is destined to become a neuron, the transition from proliferation to differentiation is accompanied by an increase in proneural *beta helix-loop-helix* (bHLH) factor expression. Specifically the bHLH factors *Neurogenin 1* and *2* and *MASH* mediate the initial stage of neurogenesis, while the neuronal differentiation factor bHLH *NeuroD* (Gao et al., 2009; Kuwabara et al., 2009) and its related gene-family drive final differentiation. Astrocyte fate is mainly guide by the activation of cytokines, in particular by the interleukin-6 family together with the activation of Notch signaling (Ehm et al., 2010). Not only does the latter promote gliogenesis, but it also inhibits neurogenesis. When neurons have migrated to their final position and are surrounded by astrocytes to form functional synapses, oligodendrocytes begin to differentiate. The sonic hedgehog (Shh) pathway (Favaro et al., 2009) is responsible for the activation of the transcriptional factors *OLIG1* and *OLIG2* which are essential to determine the oligodentrocytic fate (Doetsch et al., 2002).

Once the newly-formed cell types have reached their final location and fate, they need to be integrated into the pre-existing circuits in the adult brain. One critical factor in this process seems to be time (Mouret et al., 2008; Tashiro et al., 2006). In both the

hippocampus (Tashiro et al., 2007) and the olfactory bulb (Yamaguchi and Mori, 2005), the activity-dependent survival and integration of newborn neurons is restricted to a critical time period. Moreover, in the adult hippocampus, the integration of newborn cells appears to be coordinated by a complex neural network. Immediate early genes such as *c-fos*, *zif268*, *arc* and *homer1a* are rapidly induced by physiological stimuli and behavioral experience and are involved in the preferential incorporation of adult-born neurons into the DG neural network to support spatial memory. This process is further regulated by the neurotransmitters glutamate and GABA (Mongiat and Schinder, 2011). While the role of glutamate is still controversial, GABA is known to play a key role in the control of multiple steps of adult neurogenesis. In fact, $GABA_A$-receptors are involved in synaptogenesis and plasticity in the adult brain and contribute to proliferation, migration and differentiation of adult-born neurons (Fritschy and Panzanelli, 2014). Interestingly, in hippocampal slices, acute GABA application increases the expression of the transcription factor *NeuroD* within the SGZ (Tozuka et al., 2005). *NeuroD* is necessary to acquire a neuronal fate in the terminal differentiation stage of the granule cells in the DG (Tozuka et al., 2005). Finally, GABA-induced depolarization seems to be required for the integration of new neurons into the excitatory neuronal circuits in the hippocampus and olfactory bulb (Belluzzi et al., 2003; Tozuka et al., 2005). In addition, growing evidence supports the role of Ephrin receptors (EphRs) and their ligands in modulating neuronal synaptic structure and its physiological properties throughout lifespan (Murai and Pasquale, 2004). In juvenile rodent brains, EphRs mainly act as regulators of nervous system development by driving cell-cell interactions (Lemke, 1997). In the adult brain, EphRs are expressed in the synaptic terminals where they influence synaptic plasticity and the commitment to neuronal fate (Murai and Pasquale, 2004). Finally, the level of hippocampal neurogenesis is correlated with emotional status suggesting that the integration process highly contributes to cognitive functions (Sahay and Hen, 2007). In rodents, stress consistently decreases the adult hippocampal neurogenesis while the administration of anti-depressant increases it (Malberg et al., 2000).

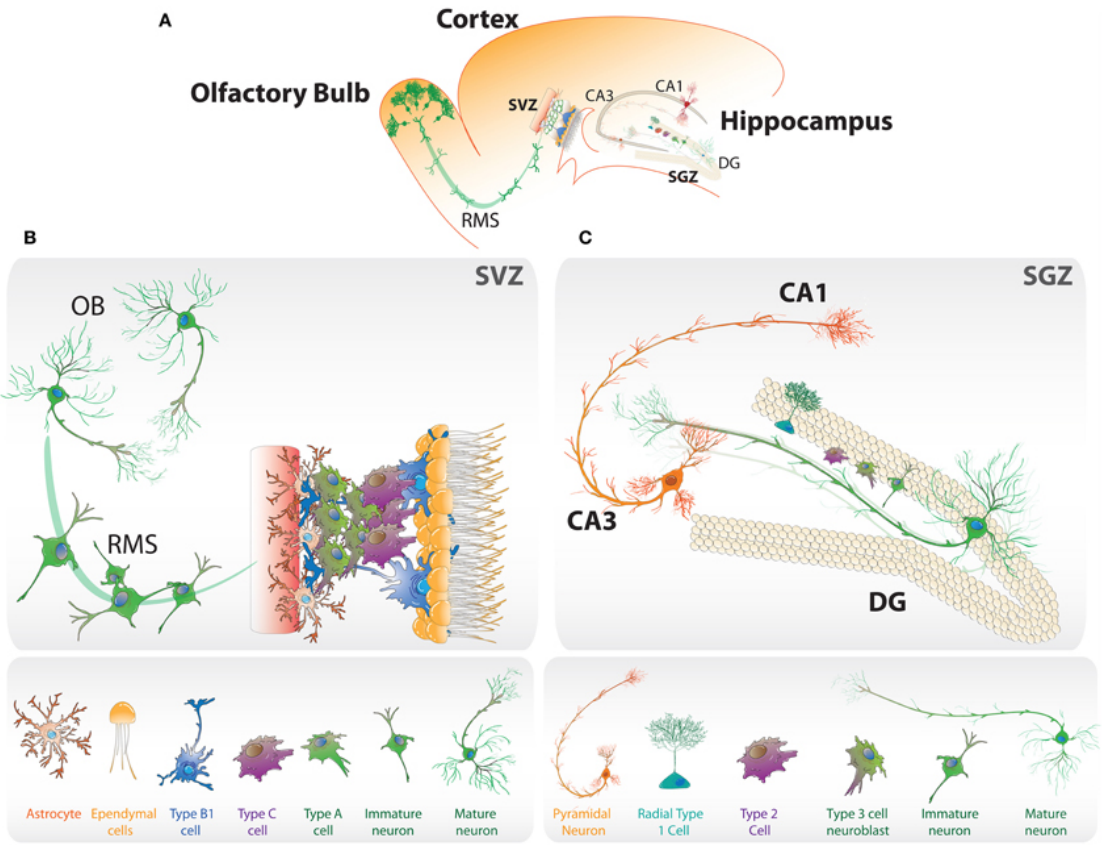


Figure 4: Established neurogenic niches in the adult brain.

(A) Schematic representation of the neurogenic regions in the adult rodent brain: the subgranular zone (SGZ) in dentate gyrus (DG) of the hippocampus, and the subventricular zone (SVZ) in the later wall of the lateral ventricles. (B) SVZ niche composed of type B1 cells, that corresponds to neural stem cells, type C cells that rapidly proliferate and type A neuroblasts, which migrate through the rostral migratory stream (RMS) to the olfactory bulb (OB) where they mature into interneurons. (C) Neurogenesis in the SGZ. Radial type 1 cells give rise to type 2 cells that differentiate into type 3 neuroblasts that become immature neurons and then mature into granule neurons that migrate into the granule cell layer (Varela-Nallar and Inestrosa, 2013).

3.3.1 Alternative neurogenic niches: the circumventricular organs

Neurogenic niches are defined as micro-environments that anatomically house stem cells and functionally control their development *in vivo*. These unique regions are characterized by certain cellular components (*i.e.,* endothelial cells, ependymal cells, astrocytes, microglia, mature neurons and progeny of adult neural precursors) and

morphology (Riquelme et al., 2008).

Worthy of note is the role of vasculature in the maintenance of the neurogenic niches. In SVZ explants from adult rats, increased neuronal differentiation is observed when explants are co-cultured with endothelial cells (Leventhal et al., 1999). Dividing cells in the adult SGZ are found to be anatomically close to the vasculature, especially capillaries (Palmer et al., 2000). Finally, in the adult SVZ, there is a broad network of planar interconnected blood vessels (Shen et al., 2008; Tavazoie et al., 2008). These findings suggest that extensive vascularity is essential in the neurogenic niches where blood-derived cues can gain direct access to adult neural precursors and their progeny.

Recently, the occurrence of neurogenesis and gliogenesis has also been demonstrated in circumventricular organs (CVOs) (Bennett et al., 2009; Hourai and Miyata, 2013; Lin et al., 2015). CVOs are midlines structures around the third and the fourth ventricles (Figure 5a). They are divided in sensory CVOs, which include the AP (Figure 5b), the OVLT, and the SFO; and secretory CVOs, which include the *median eminence* (ME), the *subcomissural organ*, the posterior pituitary and the pineal gland. CVOs are unique in structure and function. They are defined as "windows of the brain" because of their ability to directly receive information from the blood supply and produce critical brain responses in homeostatic functions (*i.e;* pH balance, blood pressure, temperature, etc.) (Gross and Weindl, 1987; Joly et al., 2007; Roth et al., 2004). This capacity is a consequence of their unique morphology: their surfaces lining the ventricles possess specialized ependymal cells called *tanycytes* that act as a partial cerebrospinal fluid barrier. Moreover, CVOs possess permeable fenestrated capillaries and a highly deficient BBB allowing them to access to important physiological stimuli. Functionally, they maintain the homeostasis of water balance, food intake and day and night rhythm (Barth et al., 2004; Brown, 1992; Mimee et al., 2013).

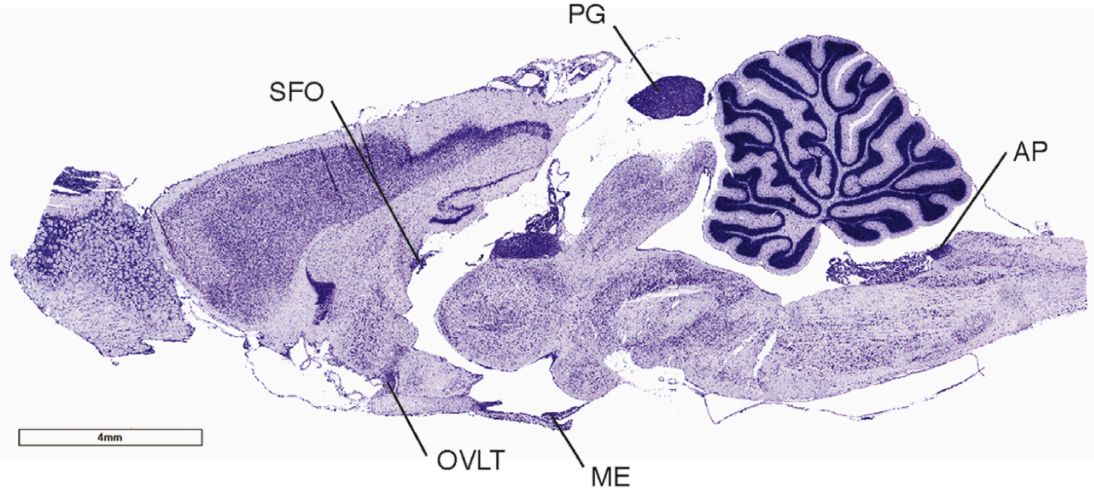
To provide an effective response to homeostatic challenges, adult CVOs have the unique capacity to continuously reconstruct their vascular and parenchymal architecture (Morita et al., 2015). This structural plasticity plays a functional role in the detection of peripheral signals and their full integration by central circuits. Particularly, the control of feeding behavior and glucose homeostasis relies on the CNS capacity to integrate diverse peripheral signals, including amylin, leptin, insulin,

glucagon, ghrelin and glucose, that reflect the nutritional and energetic state of the organism, as well as its ability to generate responses that can regulate feeding behavior, energy expenditure and the metabolic activity of cells.

In rodents, it has been shown that CVOs express neural stem cell markers, and transfected cells from CVOs proliferate and differentiate *in vitro* and *in vivo*. Interestingly, the ME, OVLT and SFO appear to be able to generate only astrocytes *in vivo*, while the AP is able to give rise to both astrocyte and neurons (Bennett et al., 2009).

At present, the functional significance of continuous neurogenesis in the adult CVOs is under investigation. Considering the peculiar nature of the CVOs, which place them in direct contact with all the substances circulating in the blood-flow, including harmful molecules, constitutive neurogenesis could primarily serve as a self-repair mechanism. Another potential explanation could be that the generation of new neurons is required to control homeostasis and the related physiological functions. In fact, it has been recently shown that in the ME, tanycytes from the ependymal zone generate new neurons that contribute to the control of food intake (Lee et al., 2012). The *de novo* formation and integration of new neurons into existing brain circuitry is part of the spectrum of plastic changes that permit the adult mammalian brain to adapt to changing functional demands. However, the exact molecular mechanisms underlying adult neurogenesis in CVOs are not yet fully understood.

a)



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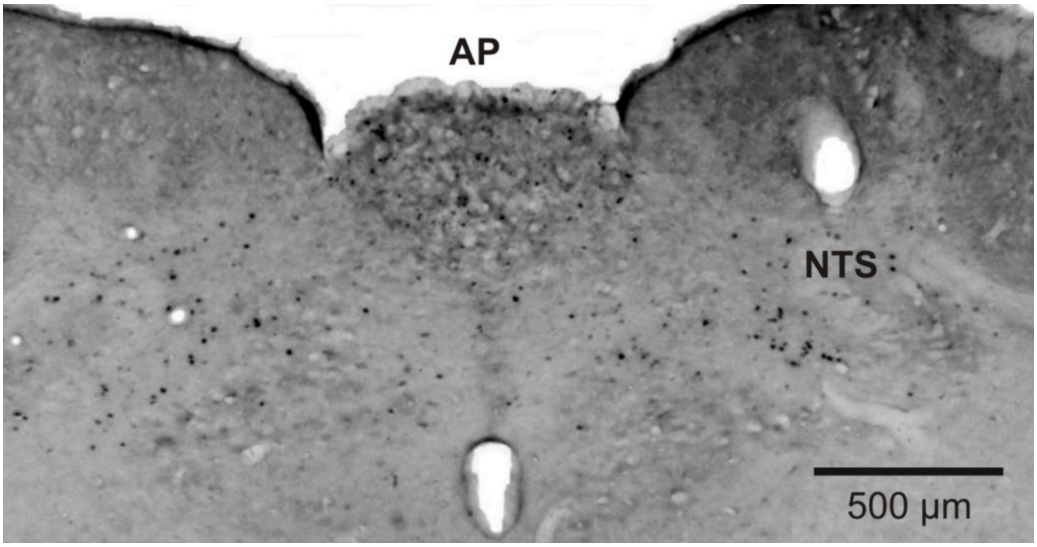

Figure 5: Distribution of the circumventricular organs

(a) Midsagittal section of the rat brain showing the distribution of the circumventricular organs (CVOs). SFO, subfornical organ; PG, pineal gland; ME, median eminence; OVLT, *organum vaculosum lamina terminalis* (Lin and Iacovitti, 2015). (b) Detailed coronal section of the rat *area postrema* (AP) and the nucleus of the solitary tract (NTS) (Edelsbrunner et al., 2009)

3.3.2 Hormones involved in the regulation of food intake and in the maintenance of energy balance play an important role in adult neurogenesis.

Adult neurogenesis is emerging as an important player in brain homeostasis and disease. In rodents, the constitutive generation of new neurons fundamentally contributes to tissue homeostasis and brain functions. Importantly, the regulation of food intake and energy balance relies on the integrity of homeostasis. Hormones that controls food intake and energy balance, such as amylin, insulin, ghrelin and leptin, seem to have an underappreciated role in adult neurogenesis (Figure 6).

The satiating hormone amylin might also exert a neuro-protective role in the brain (Adler et al., 2014). Work from our group showed that neonatal, amylin-deficient mice have reduced neuronal projections from the AP to the NTS, suggesting that amylin has the potential to function as a trophic factor in the brain (Lutz, 2010b). Moreover, in the hippocampus and AP, chronic amylin administration has been shown to compensate for the reduction in neurogenesis observed in female ovariectomized rats (Trevaskis et al, 2010). Finally, it was also shown that the chronic treatment with amylin analogue pramlintide improved memory and cognition and ameliorated hippocampal pathology in a mouse model of Alzheimer's disease (Adler et al, 2014).

Amylin is not the only hormone that plays a dual role in regulating feeding behavior and neurogenic activity. Insulin is generally known for its role in the control of food intake and energy balance. However, insulin also plays important role in cognitive functions in the brain, mainly by contributing to the regulation of structural plasticity (Chiu et al., 2008). Further, the insulin-like growth factor 1 has the ability to increase hippocampal neurogenesis and it can rescue the normal development of dendritic processes of immature neurons after brain injuries (Carlson et al., 2014).

Ghrelin is a neuropeptide hormone released by cells in the gastrointestinal tract (Inui et al., 2004; Sakata and Sakai, 2010) which regulates appetite and the distribution and rate of use of energy (Burger and Berner, 2014). Ghrelin has the ability to cross the BBB and primarily acts on hypothalamic neurons to increase hunger, gastric acid secretion and gastrointestinal motility (Schwartz et al., 2000). The ghrelin-mediated increase in appetite and fat mass is the result of the activation of a specific neuronal population in the Arc (Hewson and Dickson, 2000). Those hypothalamic neurons were identified as orexigenic Neuropeptide-Y (NPY) neurons (Dickson and Luckman,

1997) and they are responsive to ghrelin as well as to insulin and leptin (Hewson et al., 2002; Perello et al., 2012). In addition, ghrelin promotes the proliferation of intestinal cells and inhibits apoptosis during inflammatory states and oxidative stress in the gastrointestinal tract (Waseem et al., 2008). Despite its role as a hunger hormone, ghrelin also plays a role in the hippocampus where it enhances synaptic plasticity, adult neurogenesis and the maintenance of long-lasting improvements in spatial hippocampal memory (Kent et al., 2015).

In addition to its role in the control of food intake and body weight, Specifically, leptin increases adult neurogenesis *in vivo* and *in vitro* by increasing the number of proliferating cells, while cell differentiation and survival seem not to be affected by leptin treatment (Garza et al., 2008).

All these studies depict a scenario where food-related hormones may link metabolic and cognitive functions. Specifically, amylin, insulin, leptin and ghrelin might contribute to the generation and integration of new neurons into pre-existing circuits in the adult brain (Figure 5). Therefore, elucidating the underlying mechanism of this relationship holds promise to identify modifiable lifestyles factors and novel therapeutic targets that might exert beneficial effects not only on the body, but also on the brain.

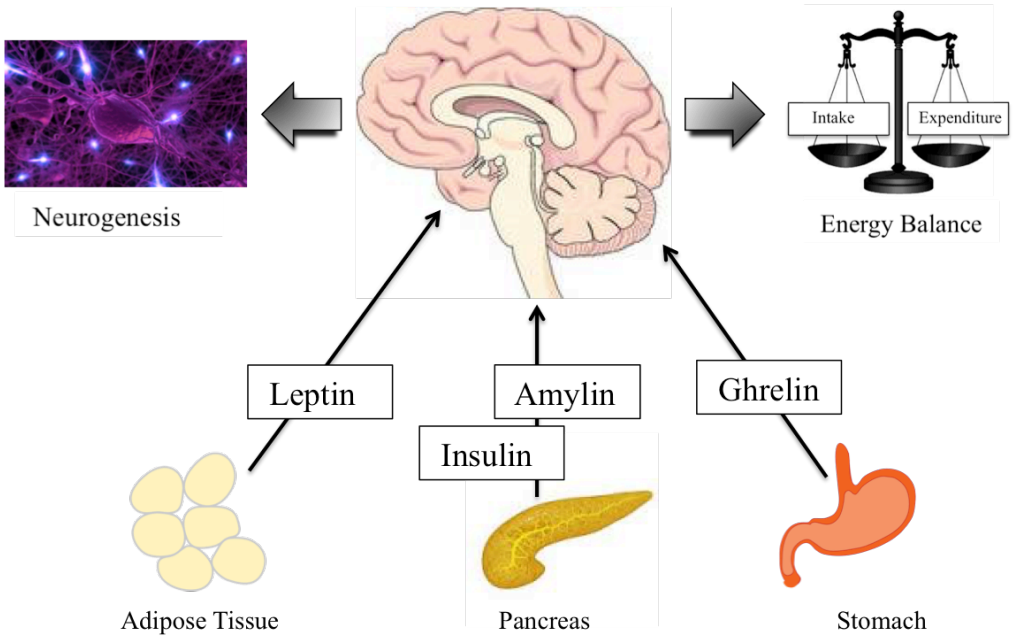

Figure 6: Hormones involved in the control of food intake contribute to the regulation of energy balance and neurogenesis.

Leptin is produced by adipose tissue, insulin and amylin are co-secreted from the pancreatic β-cells and ghrelin is released from the stomach. All these hormones participate in the coordinated regulation of food intake and the maintenance of energy balance. Leptin, insulin, amylin and ghrelin also contribute to higher cognitive functions.

Aims of the project

Aim 1: Identify the co-expression of amylin receptor subunits (CTRa, RAMP1, RAMP2 and RAMP3) and the leptin receptor b (LepRb) in single neurons in the rat *area postrema*.

A functional amylin receptor (AMY) is a heterodimer of the CTRa and one member of the RAMP family (RAMP1, RAMP2, RAMP3). The interaction between those two subunits has been described above, although the exact cellular location of these subunit remains unknown. Therefore, we evaluated the presence of the relative expression of these mRNAs in single amylin-activated neurons in the rat AP. To further assess the role of the AMY in mediating the leptin's effects, we also investigated the co-expression of the long form of the leptin receptor (Lepr-b) in single AP-neurons.

Aim 2: Analyze the transcriptome profiling of the genes differentially regulated by amylin in the rat AP.

Previous findings (Hindmarch et al., 2011) partially describe the pool of genes present in the rat AP, but the transcripts differentially regulated by amylin have not been investigated and their biological relevance remains unclear. Next-generation sequencing allows for a full characterization of the transcriptome of the AP. To describe the gene expression of the rat AP, we injected animals with vehicle, amylin or with amylin and the amylin antagonist AC187 injections.

Aim 3: Investigate the role of amylin in adult neurogenesis in the rat AP.

CVOs have been recently described as new "neurogenic niches" in the adult mammalian brain. The AP is a well-known sensory CVO which monitors blood-derived information and relaying this information to other brain regions. Neurogenesis and gliogenesis have been demonstrated in the AP *in vivo;* moreover, recently amylin has been shown to promote the formation of neuronal projections originating from the neonatal mouse AP. However, the role of amylin in adult neurogenesis remains unknown. Here we evaluated the effect of chronic amylin infusion on the number of proliferative cells and their cell fates in the AP of adult rats.

Aim 4: Characterize the feeding behavior of the *Nestin/hRamp1* transgenic mouse model which overexpresses RAMP1.

Previous work (Zhang et al., 2011) demonstrates that *Nestin/hRAMP1* double transgenic mice are characterized by a marked lean phenotype associated with increased EE and normal food intake. This suggests, that the overexpression of RAMP1 might exert a protective role against obesity. However, a full characterization of feeding behavior in these mice on chow is still missing. Therefore, we monitored body weight, food intake, meal patterns, meal size and energy expenditure of *Nestin/hRAMP1* mice maintained on normal chow. To further assess whether RAMP1 can protect mice from diet-induced obesity, we also challenged the animals with HFD).

5. Original Research article: “Amylin receptor components and the leptin receptor are co-expressed in single area postrema neurons”

This section contains an original research article that was submitted for publication to the European Journal of Neuroscience in September 2015 and accepted for publication in revised form in December 2015.

My contribution to this manuscript includes the study design, data acquisition, data analysis, data interpretation and drafting and revising the manuscript.

EJN European Journal of Neuroscience

FENS Federation of European Neuroscience Societies

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Amylin receptor components and the leptin receptor are co-expressed in single rat area postrema neurons

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Keywords: calcitonin receptor, laser capture microdissection, receptor activity-modifying protein 1, receptor activity-modifying protein 2, receptor activity-modifying protein 3

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Abstract

Amylin is a pancreatic β-cell hormone that acts as a satiating signal to inhibit food intake by binding to amylin receptors (AMYs) and activating a specific neuronal population in the area postrema (AP). AMYs are heterodimers that include a calcitonin receptor (CTR) subunit [CTR isoform a or b (CTRa or CTRb)] and a member of the receptor activity-modifying proteins (RAMPs). Here, we used single-cell quantitative polymerase chain reaction to assess co-expression of AMY subunits in AP neurons from rats that were injected with amylin or vehicle. Because amylin interacts synergistically with the adipokine leptin to reduce body weight, we also assessed the co-expression of AMY and the leptin receptor isoform b (LepRb) in amylin-activated AP neurons. Single cells were collected from Wistar rats and from transgenic *Fos-GFP* rats that express green fluorescent protein (GFP) under the control of the Fos promoter. We found that the mRNAs of CTRa, RAMP1, RAMP2 and RAMP3 were all co-expressed in single AP neurons. Moreover, most of the CTRa+ cells co-expressed more than one of the RAMPs. Amylin down-regulated RAMP1 and RAMP3 but not CTR mRNAs in AMY+ neurons, suggesting a possible negative feedback mechanism of amylin at its own primary receptors. Interestingly, amylin up-regulated RAMP2 mRNA. We also found that a high percentage of single cells that co-expressed all components of a functional AMY expressed LepRb mRNA. Thus, single AP cells expressed both AMY and LepRb, which formed a population of first-order neurons that presumably can be directly activated by amylin and, at least in part, also by leptin.

Introduction

Amylin, also known as islet amyloid polypeptide, is co-secreted with insulin by pancreatic β-cells in response to nutrient stimuli (Lutz, 2010). Amylin reduces food intake and body weight (Lutz *et al.*, 2001; Roth *et al.*, 2012) and may also act as an adiposity signal to control energy expenditure (Wielinga *et al.*, 2007; Zhang *et al.*, 2011). Circulating amylin acts centrally to control the energy balance by primarily activating neurons of the area postrema (AP), a circumventricular organ located in the hindbrain (Riediger *et al.*, 2001, 2004; Lutz, 2009; Potes & Lutz, 2010; Potes *et al.*, 2012).

A functional amylin receptor (AMY) results from a heterodimer of the calcitonin receptor (CTR) with one member of the receptor activity-modifying proteins (RAMPs) (Christopoulos *et al.*, 1999). The rat CTR exists in two different isoforms, CTRa and CTRb, but the exact functional relevance of action mediated by either isoform is not yet fully understood. *In situ* hybridization studies that mapped the localization of CTRa/b and RAMPs suggested that only CTRa is present in the AP of rodents (Ueda *et al.*, 2001; Barth *et al.*, 2004). Three members of the RAMP family have been identified (McLatchie *et al.*, 1998; Sexton *et al.*, 2001): RAMP1, RAMP2 and RAMP3. They are associated in the endoplasmic reticulum and are co-trafficked to the cell surface in order to form stable complexes that act as chaperones to form different receptors with selective ligand specificity. The dimerization of RAMP1, RAMP2 and RAMP3 with CTRa generates AMY_1, AMY_2 and AMY_3, respectively (Bailey *et al.*, 2012; Alexander *et al.*, 2013).

The presence of CTR and RAMPs has been shown in different brain areas (Sexton *et al.*, 1994; Christopoulos *et al.*, 1995; Skofitsch *et al.*, 1995; Becskei *et al.*, 2004; Mietlicki-Baase *et al.*, 2013). However, none of these studies tested the co-localization of the AMY components at the single-cell level, which is necessary to study the physiological relevance of CTR and RAMPs *in vivo*.

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Amylin is also known to synergistically interact with leptin to decrease body weight and food intake (Roth *et al.*, 2008; Trevaskis *et al.*, 2010). Leptin exerts its effects by binding to the leptin receptor isoform b (LepRb), and the ventromedial nucleus of the hypothalamus may be the site mediating the amylin–leptin interaction (Trevaskis *et al.*, 2010; Turek *et al.*, 2010; Le Foll *et al.*, 2015). Other brain areas, such as the AP (Roth *et al.*, 2008; Trevaskis *et al.*, 2010) or ventral tegmental area may also play a role (Mietlicki-Baase *et al.*, 2015); however, a population of first-order neurons bearing receptors for both amylin and leptin has yet to be reported (Turek *et al.*, 2010; but see Smith *et al.*, 2015). Evidence of single neurons expressing both hormone receptors is lacking.

To establish that all components of the AMY are present in single AP cells and to investigate whether AP neurons co-express AMY and leptin receptors, we examined the mRNA expression of CTRa and RAMPs in single AP cells, and of LepRb in AMY+ neurons.

Materials and methods

Animals and tissue collection

Male Wistar rats (Janvier, Le Genest Saint Isle, France) (Experiments 1 and 2; 200–225 g) and male and female *Fos-GFP* rats (NIDA/NIH; Baltimore, MD, USA and NIMH/NIH; Bethesda, MD, USA) (Experiments 3–5; 250–320 g) were single-housed in a temperature-controlled environment (21 ± 1 °C) on an artificial 12-h/12-h light/dark cycle. Rats had *ad libitum* access to water and standard chow, except during fasting periods as described below. All procedures involving animals and their care were approved by the Veterinary Office of the Canton Zürich, Switzerland, and were in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

Drugs

Amylin (Bachem AG, Bubendorf, Switzerland; catalogue no. H-9475.1000) was reconstituted with sterile 0.9% NaCl.

Experimental design

Rat injections and sample preparation

For all experiments, rats were fasted for 12 h and injected i.p. with vehicle or amylin (20 μg/kg for Wistar rats and 50 μg/kg for *Fos-GFP* rats) at dark onset. At 90 min after drug administration, rats were anaesthetized with isoflurane and decapitated.

Experiment 1

Brains from Wistar rats ($n = 4$) were promptly processed under a light microscope. The AP was surgically removed from the brainstem and then the subfornical organ (Takahashi *et al.*, 1997) and hypothalamus were dissected. All of the tissues were placed immediately in TRI Reagent® (Sigma-Aldrich) and processed for reverse transcription-polymerase chain reaction, as described below.

Experiments 2–5

Brains were rapidly isolated, snap-frozen in cold isopentane and embedded in Tissue-Tek® OCT™ (Sakura Finetek) compound just prior to sectioning. Coronal sections were cut at 10-μm thickness on a cryostat (Leica, Germany) through the entire AP and affixed on precleaned, Superfrost glass slides (Thermo Scientific, Waltham, MA, USA). The sections were immediately placed in a slide box on dry ice until completion of sectioning. The slide box was rinsed with RNase Away (Molecular BioProduct, Mexico) prior to usage to prevent RNA degradation. Samples were then stored at −80 °C until the laser capture microdissection (LCM) procedure.

In Experiment 2, Wistar rats ($n = 4$/group) were treated with amylin (20 μg/kg, i.p.) and single neurons ($n = 8$–10 cells/rat) were identified with cresyl violet staining as previously described (Kadar *et al.*, 2009). CTRa, RAMP1 and RAMP3 have been extensively described as being present in the rat AP (Becskei *et al.*, 2004), thus generating AMY_1 and AMY_3 as the primary AMYs in this brain nucleus. RAMP2 mainly contributes to the generation of AMY_2 by coupling with CTRb (Tilakaratne *et al.*, 2000). Because CTRb is not expressed in the rat AP (Ueda *et al.*, 2001; Barth *et al.*, 2004), we mainly focused on the analysis of RAMP1 and RAMP3 in combination with CTRa.

In Experiments 3–5, *Fos-GFP* transgenic male and female rats ($n = 4$ rats/treatment/gender) were treated with vehicle or amylin (50 μg/kg, i.p.). *Fos-GFP* rats express the green fluorescent protein (GFP) under the *Fos* promoter in strongly activated neurons (Cifani *et al.*, 2012) and therefore no additional immunohistochemical procedure was required to visualize *Fos-GFP*-activated neurons. Amylin-stimulated cells ($n = 9$–10 cells/rat) were readily detectable under the enhanced GFP (eGFP) filter (490–560 nm).

Reverse transcription-polymerase chain reaction

RNA was extracted according to the manufacturer's instructions (TRI Reagent®, Sigma-Aldrich) and then purified following the clean-up protocol of the RNeasy® Mini kit (Qiagen, Germany), including the DNase step. The concentration and integrity of RNA were measured using a nanodrop system (NanoDrop 1000 Spectrophotometer, Thermo Scientific). cDNA was generated from the extracted RNA using the Terto cDNA synthesis kit (Bioline, Switzerland).

The primer pairs for rat CTRa/b were reported previously (Mori *et al.*, 2006) and the primers for rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were manually designed using Primer 3 web version 3.0.0. The primer sequences for rat CTRa/b were as follows: forward: 5′-TGGTTGAGGTTGTGCCCAATGGA-3′ and reverse: 5′-TCCATGGGTTTGCCTCATCTTGGTC-3′. This primer pair was able to differentiate the two isoforms of CTR, giving a 392-bp product for CTRa and a 503-bp product for CTRb (Accession no. L14617 and L13040). For rat GAPDH the primer sequences were as follows: forward: 5′-GCCAGCCTCGTCTCATA GACA-3′ and reverse: 5′-GTGCGATACGGCCCAATC-3′ (Accession no. NM_017008.4). The reverse transcription-polymerase chain reaction conditions were as follows: initial denaturation for 3 min at 95 °C, followed by 35 alternating cycles of 15 s at 95 °C and 15 s at 60 °C, and a final extension step of 20 s at 72 °C. The reverse transcription-polymerase chain reaction amplification products were separated by 2% agarose gel electrophoresis (3000 Xi, BioRad).

Laser capture microdissection

Slides containing AP sections, collected from Wistar rats and vehicle-treated *Fos-GFP* animals, were Nissl stained to allow cell identification. Slides were removed from −80 °C and thawed at 4 °C for 2 min, and sections were stained in 0.1% cresyl violet acetate (Sigma-Aldrich) solution dissolved in 70% ethanol for 5 min on ice. The excess stain was drained on filter paper. Sections were then

dehydrated once in 96% EtOH and once in 100% EtOH, for 30 s each, on ice, and finally dried in a fume hood for 2 min at room temperature (21 ± 1 °C) before being processed under the LCM microscope (Arcturus X MDS, Life Technologies, Carlsbad, CA, USA). As described above, no additional immunohistochemical procedure was required to identify *Fos-GFP*+ cells in *Fos-GFP* transgenic rats. Brain slices were observed under the microscope and the AP was localized based on morphology (Bregma −13.68 to −14.28 mm; Paxinos & Watson, 2007).

In Wistar rats and vehicle-treated *Fos-GFP* rats, single cells were visualized with Nissl staining, randomly selected and individually collected in caps (CapSure® HS LCM caps, Arcturus Bioscience). In amylin-treated transgenic rats, single *Fos-GFP*+ cells were identified under the eGFP filter and collected in individual caps. Total RNA was extracted and isolated, including a DNase step, using the vendor's protocol (PicoPure™ RNA isolation kit, Arcturus Bioscience) and subsequently stored at −80 °C.

Quantitative polymerase chain reaction

RNA extracted from each individual cell was amplified using Ribo-SPIA technology (Ovation One-Direct System, part no. 3500; NuGEN, San Carlos, CA, USA) and cDNA was subsequently purified using Agencourt RNAClean XP beads. A post-SPIA modification process was run to complete the process and to generate sense-target cDNA, according to the manufacturer's instructions. Single-cell quantitative polymerase chain reaction was performed using the 7500 Fast system (Applied Biosystem/Life Technologies) with the QuantiTect® SYBR® green polymerase chain reaction kit (Qiagen). Rat GAPDH was used as the housekeeping gene. To avoid the possibility of amplifying contaminating DNA, intron-spanning primer pairs for rat CTRa, RAMP1 and RAMP3 were designed with Primer 3 web version 4.0.0. The primer sequences were as follows: for rat GAPDH, forward: 5′-AGACAGCCGCATCTTCTTGT-3′ and reverse: 5′-CTTGCCGTGCGTAGAGTCAT-3′ (Accession no. NM_017008.4); for rat CTRa, forward: 5′-TTTCCAGGGATTCT TTGTCG-3′ and reverse: 5′-TTCGTTGCTGACTGGAG-3′ (Accession no. NM_001034015.1); for rat RAMP1, forward: 5′-GGCAAA CAAGATTGGCTGTT-3′ and reverse: 5′-AATGGGGAGCACAAT GGAAG-3′ (Accession no. NM_031645.1); for rat RAMP2, primer pairs were previously reported by Qi *et al.* (2003), forward: 5′-TGAGGACAGCCTTCTGTCAA-3′ and reverse: 5′-CATCGCCG TCTTTACTCCTC-3′ (Accession no. NM_031646.1); and for rat RAMP3, forward: 5′-AGGTCATCTGGAAGGTGTGG-3′ and reverse: 5′-AATGGGGAGCACAATGGAAG-3′ (Accession no. NM_02100.2). The intron-spanning primer pairs for rat LepRb were designed with Integrated DNA Technologies as follows: forward: 5′-GGTTGGATGGACTAGGGTATTG-3′ and reverse: 5′-CAGAAT TCAGGCCCTCTCATAG-3′ (Accession no. NM_012596.1).

A total of 200 ng of cDNA was subjected to an initial heat activation at 95 °C for 15 min, followed by 40 cycles alternating between 94 °C for 15 s and 60 °C for 30 s, and final extension at 72 °C for 30 s. The relative transcriptional levels of CTRa, RAMP1, RAMP2, RAMP3 and LepRb mRNA were calculated using the comparative ΔΔCt method that generates relative CTRa, RAMP1 and RAMP3 mRNA levels adjusted for the GAPDH endogenous control mRNA. Each sample was run in duplicate.

Statistical analysis

The CTRa+ cells vs. CTRa− cells (n = 4 rats, 8–10 cells/rat) and the related presence or absence of RAMPs, respectively, were analysed with the Chi-squared test (Experiment 2). Relative mRNA expression levels were assessed by unpaired Student's t-test (Experiment 4: n = 4 rats, 9–10 cells/rat; Experiment 5: n = 4 rats, 8–10 cells/rat) by using GRAPHPAD software version 5.0 (San Diego, CA, USA).

Results

Experiment 1: only the CTRa isoform was expressed in native rat area postrema tissue

Confirming previous studies (Hilton *et al.*, 1995; Barth *et al.*, 2004), we observed that only the CTRa isoform was expressed in native rat AP tissue (Fig. 1). The subfornical organ and hypothalamus were used as positive controls, with both brain regions expressing CTRa and CTRb isoforms (Becskei *et al.*, 2004; Mori *et al.*, 2006).

Experiment 2: randomly collected cells from the area postrema of amylin-treated rats presented a complex neuronal landscape. Most of the CTRa+ cells were RAMP1+ and RAMP3+, whereas most of the CTRa− cells were also RAMP1− and RAMP3−

The rapid Nissl staining procedure before LCM enabled the identification of individual cells in the AP sections of amylin-treated Wistar rats (Fig. 2a). Cells were randomly selected and individually collected by LCM (Fig. 2a, inset). A presence/absence assay was performed in which GAPDH was used as the endogenous control and the analysed neuronal population was divided into CTRa+ and CTRa− cells, respectively. The resulting neuronal landscape showed a complex scenario (Fig. 2b). Almost 40% of the collected neurons were CTRa+ cells; importantly, all CTRa+ cells co-expressed CTRa and RAMP1 (3%), CTRa and RAMP3 (10%) or CTRa and both RAMP1 and RAMP3 (25%); 62% of the randomly collected cells were CTRa−. Moreover, most of the CTRa− cells were also RAMP1− and RAMP3− (53%); only 9% of CTRa− cells showed either RAMP1 (6%) or RAMP3 (3%) expression. Hence, RAMP1 and RAMP3 mRNAs were preferentially expressed in CTRa+ vs. CTRa− cells [Chi-squared test (30.13, 3), $P < 0.0001$]. The key finding of this study was that CTRa+ cells co-expressed RAMP1 and/or RAMP3 transcript, which are the necessary subunits required for a specific and functional AMY_1 or AMY_3.

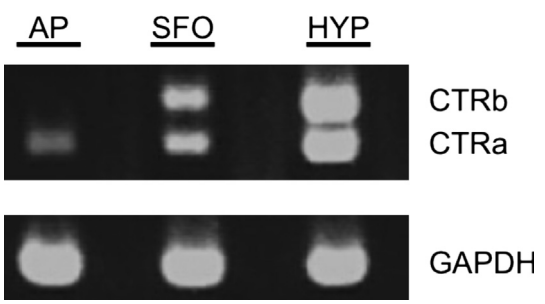

FIG. 1. Reverse transcription-polymerase chain reaction showing the distribution of CTR isoforms in different brain nuclei. Agarose gel electrophoresis of CTRa (392 bp) and CTRb (503 bp) polymerase chain reaction products from rat AP, subfornical organ (SFO) and hypothalamus (HYP). GADPH (207 bp) was used as the reference gene and run in different lanes. Only the CTRa is detected in the rat AP.

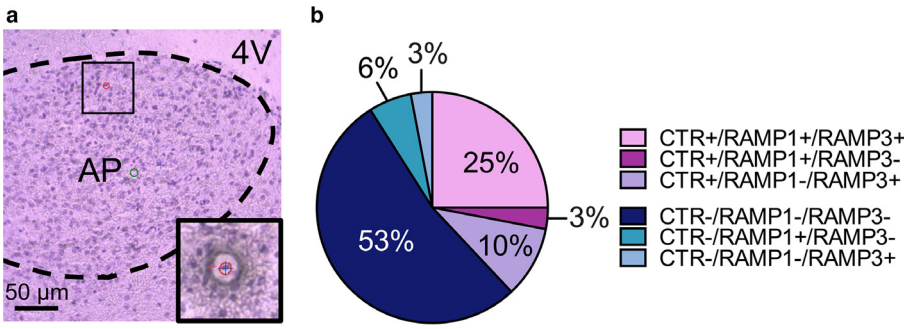

FIG. 2. Distribution of CTR, RAMP1 and RAMP3 mRNAs in single cells of amylin-treated rats. (a) Nissl staining of rat AP at 20 ×. Representative image showing the LCM laser (blue cross) in the act of collecting a single neuron (red circle with number 1). The green circle represents the ultraviolet laser cutting spot. Scale bar in inset: 12.5 µm. (b) In randomly picked individual neurons (n = 8–10 cells/rat) from the AP of amylin-treated (20 µg/kg) male Wistar rats (n = 4), 38% of individual neurons are CTRa+ [25% co-express CTRa and both RAMP1 and RAMP3; 3% co-express CTRa and only RAMP1 and 10% co-expressed CTRa and only RAMP3]. The remaining 62% of the randomly collected single cells are CTRa−; most of the CTRa− cells are also RAMP1− and RAMP3− (53%); 6% of all cells are CTRa−, RAMP1+ and RAMP3−, and 3% of all cells are CTRa−, RAMP1− and RAMP3+. RAMP1 and RAMP3 transcripts are preferentially expressed in CTRa+ vs. CTRa− neurons. Chi-square test, $P < 0.001$. 4 V, fourth ventricle.

Experiment 3: CTRa, RAMP1 and RAMP3 mRNAs were all co-expressed in the same single amylin-activated area postrema neurons of Fos-GFP rats

Results from Experiment 2 clearly showed that, in the neuronal population defined as CTRa+, single cells co-expressed RAMP1, RAMP3 or both RAMP1 and RAMP3 mRNAs in addition to CTRa. We reasoned that only this pool of cells expressed a biologically functional AMY_1 or AMY_3 and therefore could be activated by amylin. To test this hypothesis, we combined the use of *Fos-GFP* transgenic rats and single-cell quantitative polymerase chain reaction to better characterize the transcripts of CTRa, RAMP1 and RAMP3 in amylin-activated individual neurons.

Transgenic rats were treated with vehicle or amylin (Fig. 3a and d, respectively). In the control group, single cells were visualized with Nissl staining and randomly collected. Results obtained from the randomly collected single cells treated with vehicle replicated the scenario of Experiment 2; we confirmed that CTRa− cells were also generally RAMP1− and RAMP3− (50% of all cells investigated in male rats and 33% in female rats, respectively), whereas CTRa+ cells were also RAMP1+, RAMP3+ or both RAMP1+ and RAMP3+ (see Fig. 3b and c for the detailed distribution in male and female rats, respectively). Amylin treatment induced strong *Fos-GFP* expression in the AP (Fig. 3d), allowing the specific collection of single amylin-activated neurons. In male rats, 64% of the cell population showed co-expression of CTRa, RAMP1 and RAMP3 in the same single neuron; 9% of the collected single neurons co-expressed CTRa with either RAMP1 (1%) or RAMP3 (8%). Only 27% of the neuronal population expressed one of the RAMPs but not the CTR (for details, see Fig. 3e). Results obtained from female transgenic rats displayed a pattern that was similar to the males. In the amylin-treated female rats, the entire population of amylin-responsive cells showed co-expression with CTRa and RAMPs; 70% of the collected single cells were CTRa+, RAMP1+ and RAMP3+, whereas the remaining 30% showed co-expression of CTRa with either RAMP1(10%) or RAMP3 (20%) (Fig. 3f).

Experiment 4: amylin down-regulated RAMP1 and RAMP3 transcripts in single area postrema neurons, whereas RAMP2 mRNA was up-regulated

To provide an absolute quantification of the expression levels of the mRNAs of CTRa and RAMPs, we selected all of the CTRa+ cells and compared the relative expression of RAMP1, RAMP2 and RAMP3 in control-treated and amylin-treated *Fos-GFP* transgenic rats. RAMP2 mRNA was also included in this experiment to test specifically if identified CTRa+ cells also expressed RAMP2, in addition to RAMP1 and RAMP3.

Our findings demonstrated that, whereas CTRa expression seemed to be unaffected by acute amylin treatment ($t_{79} = 0.3573$, $P = 0.3573$), RAMP1 and RAMP3 mRNAs were down-regulated in amylin-activated single neurons (RAMP1: $t_{62} = 5.409$, $P < 0.0001$; RAMP3: $t_{67} = 6.640$, $P < 0.0001$; Fig. 4b and d). Interestingly, amylin up-regulated RAMP2 mRNA in single AP cells ($t_{34} = 3.832$; $P < 0.0001$, Fig. 4c). See Table 1 for Cycle Threshold (CT) values.

To evaluate the co-expression of RAMP2 mRNA relative to RAMP1 and RAMP3, a presence/absence analysis was performed in all CTRa+ cells. In vehicle-treated animals, 70% of single CTRa+ AP neurons expressed RAMP2+ in addition to RAMP1 (2%), RAMP3 (13%) or both RAMP1 and RAMP3 (55%; Fig. 5a); none of the CTRa+ cells expressed only RAMP2. The RAMP2 mRNA distribution was relatively similar in amylin-treated animals (for details, see Fig. 5b). These findings provide additional information on the distribution of RAMP2 mRNA relative to the other RAMPs.

Experiment 5: 30% of amylin-treated single area postrema neurons that co-expressed all of the necessary components to form a functional amylin receptor also co-expressed LepRb mRNA

The presence of LepRb mRNA was investigated in the rats from Experiment 3 in single cells that were found to express all of the necessary components to form a functional AMY (CTRa+ and either individual or combined RAMPs were defined as AMY+). Single AMY+ cells from both vehicle-treated and amylin-treated groups were analysed. Under vehicle conditions, 52% of single AMY+ cells also co-expressed LepRb mRNA (Fig. 6a). In rats treated with amylin, 30% of the AMY+ single cells also co-expressed LepRb (Fig. 6b). Interestingly, the relative mRNA level of LepRb in AMY+ cells was significantly up-regulated by amylin treatment ($t_9 = 2.286$, $P < 0.05$; Fig. 6c; see Table 1 for CT values).

Discussion

The CTR and RAMPs generate receptors with high affinity for amylin (Christopoulos *et al.*, 1999), with the precise nature of these

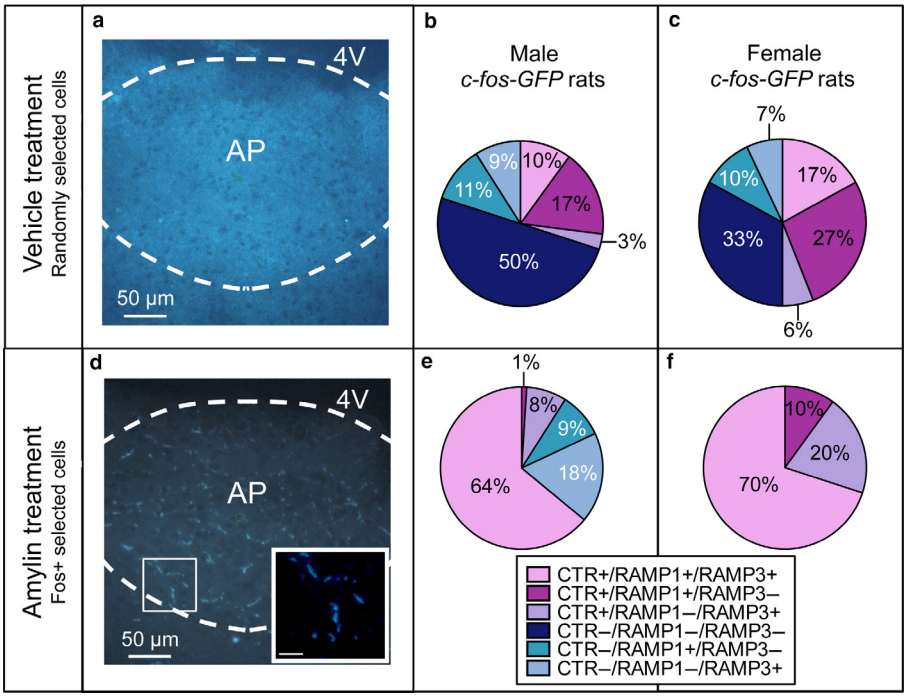

FIG. 3. Analysis of single cells captured from vehicle and amylin-treated *Fos-GFP* rats. AP sections of *Fos-GFP* rats. (a) Vehicle-treated male rats (n = 4/group/gender). (b) The randomly collected single cells (n = 9–10 cells/rat) show a mixed distribution: 30% of the single cells were CTRa+/RAMPs+; specifically 10% were CTRa+/RAMP1+/RAMP3+, 17% were CTRa+/RAMP1+/RAMP3− and 3% were CTRa+/RAMP1−/RAMP3+. In the remaining cell population, 50% of the neurons were CTRa−/RAMP1−/RAMP3−, 11% were CTRa−/RAMP1+/RAMP3− and 9% were CTRa−/RAMP1−/RAMP3+. (c) In *Fos-GFP* female rats, the results in the vehicle-treated group were consistent with what was shown in males, i.e. 50% of the collected single cells were CTRa+/RAMPs+; specifically, 17% were CTRa+/RAMP1+/RAMP3+, 27% were CTRa+/RAMP1+/RAMP3− and 6% were CTRa+/RAMP1−/RAMP3+. The remaining cell population was characterized as CTRa−, 33% being CTRa−/RAMP1−/RAMP3−, 10% being CTRa−/RAMP1+/RAMP3− and 7% being CTRa−/RAMP1−/RAMP3+. (d) Amylin-induced Fos expression in AP neurons. The inset shows examples of single amylin *Fos-GFP* activated cells (scale bar: 10 µm). The green circle represents the ultraviolet cutting spot. (e) In *Fos-GFP* male rats, the 73% of neurons that showed *Fos-GFP* expression after amylin injection (50 µg/kg) were CTRa+. Most of the CTRa+ cells (64%) co-expressed the mRNA of CTRa, RAMP1 and RAMP3, 1% were CTRa+/RAMP1+/RAMP3− and 8% were defined as CTRa+/RAMP1−/RAMP3+. The remaining neuronal population was characterized as CTR−/RAMP1+/RAMP3− (9%) and CTR−/RAMP1−/RAMP3+ (18%). (f) In amylin-treated *Fos-GFP* female rats, all *Fos-GFP* activated neurons were CTRa+/RAMPs+; 70% of the population co-expressed CTRa, RAMP1 and RAMP3 in the same single cell, and the remaining 30% co-expressed CTRa and RAMP1 (10%) or CTRa and RAMP3 (20%). 4 V, fourth ventricle.

receptors depending on the CTR splice variant and the cellular background. In the present study we provide evidence that the mRNA for all components of the AMY complex is co-expressed in the same single neurons in the rat AP, suggesting intracellular interactions between these subunits to mediate amylin signalling in the brain. This is the first demonstration in native tissue of single cells expressing all components of the functional AMY that allows them to be activated directly by amylin. We confirmed that only the CTRa isoform of the CTR is expressed in the AP; therefore, the AMY complex in this brain nucleus results from a dimer of one or more RAMPs exclusively with CTRa (Barth *et al.*, 2004; Becskei *et al.*, 2004).

By applying the LCM technique, we were able to fully characterize the presence of functional AMY at the single-cell level. Our results indeed support a scenario where in basal conditions AP neurons stochastically express zero, one, two, three or four components of the AMY. However, only the neurons that concurrently express CTRa and at least one of the RAMPs bear a functional AMY, and therefore have the full potential to be directly activated by amylin.

Previous findings demonstrated that RAMP1, RAMP2 and RAMP3 all interact with CTRa and CTRb to generate functional $AMY_{1–3}$ *in vitro* (McLatchie *et al.*, 1998; Christopoulos *et al.*, 1999; Hay *et al.*, 2004). Different CTR/RAMP complexes have distinct pharmacology, which may determine the rate of amylin binding to a specific AMY subtype. AMY_1 and AMY_3 have high affinities for amylin and salmon calcitonin and both have the potential to bind the calcitonin gene-related peptide. However, AMY_1 has a 30-fold higher affinity for calcitonin gene-related peptide than AMY_3 (Christopoulos *et al.*, 1999), implying that calcitonin gene-related peptide is most likely to target AMY_1 rather than AMY_3. It is important to mention that, in particular, the ability of RAMP2 to form functional AMY_2 is clearly dependent on the cellular background and on the receptor isoform (Tilakaratne *et al.*, 2000). In fact, CTRb displayed greater capacity to dimerize with RAMP2 to generate a functional AMY_2 than CTRa (Morfis *et al.*, 2008). Given that CTRb is not present in the AP, our work mainly focused on the characterization and co-expression of CTRa, RAMP1 and RAMP3.

Our findings showed that, in both male and female rats, the majority (over 60%) of the amylin-activated, *Fos-GFP*+ single cells co-expressed CTRa, RAMP1 and RAMP3 and a smaller percentage co-expressed CTRa and either RAMP1 or RAMP3. This suggests that amylin mainly activates cells that express mRNA for CTRa, RAMP1 and RAMP3. When CTRa was co-expressed with only one of the RAMPs, the accessory subunit was preferentially RAMP3 (for details, see Fig. 3e and f), suggesting that AMY_3 might be the primary AMY at the cellular level for AP-mediated amylin actions. The preferential expression of CTRa with RAMP1 and/or RAMP3 in these activated cells also supports the idea that AMY_1 and AMY_3 are the most physiologically relevant receptors mediating amylin actions in the AP, and indicates that both subtypes can be present in

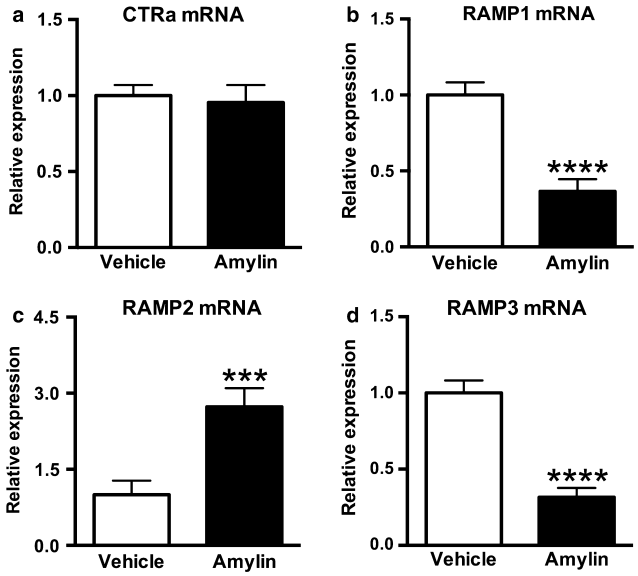

FIG. 4. Effect of amylin on the transcriptional expression of CTRa, RAMP1, RAMP2 and RAMP3 mRNAs in AP single cells. In *Fos-GFP* female rats (n = 4/group), AP single neurons (n = 9–10 cells/rat), which showed co-expression of CTRa and RAMPs in both vehicle-treated and amylin-treated animals, were analysed by quantitative polymerase chain reaction. mRNA expression is shown as fold change; rat GAPDH was used as the housekeeping gene to normalize the data. (a) CTRa mRNA levels were unaffected by the experimental condition, whereas (b) RAMP1 and (d) RAMP3 transcripts were significantly down-regulated in amylin-treated animals compared with control-treated rats (RAMP1, ****$P < 0.001$; RAMP3, ****$P < 0.001$). (c) Amylin up-regulated RAMP2 mRNA compared with control (***$P < 0.001$); Data shown as mean + SEM.

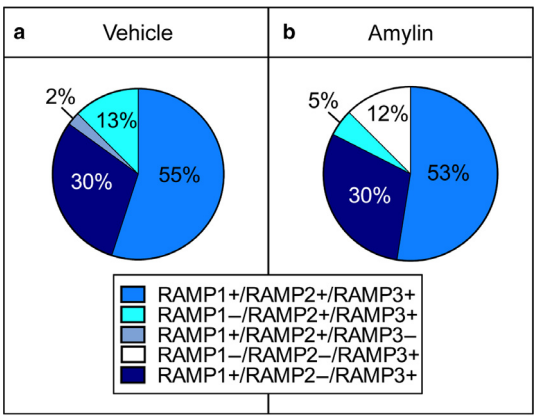

FIG. 5. Distribution of RAMP2 mRNA in relation to the other RAMPs. All of the selected cells were CTRa+. (a) In vehicle *Fos-GFP* female rats (n = 4/group), the collected AP single neurons (n = 9–10 cells/rat) were mainly characterized as RAMP2+, being RAMP1+/RAMP2+/RAMP3+ (55%), RAMP1−/RAMP2+/RAMP3+ (13%), RAMP1+/RAMP2+/RAMP3− (2%) and RAMP1+/RAMP2−/RAMP3+ (30%). (b) RAMP2 mRNA distribution in amylin-treated animals was as follows: RAMP1+/RAMP2+/RAMP3+ (53%), RAMP1−/RAMP2+/RAMP3+ (5%); RAMP1−/RAMP2−/RAMP3+ (12%) and RAMP1+/RAMP2−/RAMP3+ (30%).

individual AP neurons. In *Fos-GFP* male animals, 27% of amylin-activated, *Fos-GFP*+ neurons were characterized as CTR− but expressed either RAMP1 (9%) or RAMP3 (18%). It is unlikely that these cells were directly activated by amylin but, because they expressed GFP after amylin administration, they possibly represent second-order neurons.

The congruent results obtained in males and females suggest that the sex of the rat does not seem to be an important factor influencing the genetic expression of the components of the AMY. However, sex hormones may still influence the relative expression of the AMY components. Oestradiol strongly up-regulates the mRNA levels of RAMP3 in the rat uterus (Watanabe *et al.*, 2006), but there have been no investigations into whether oestradiol may have similar effects in the brain. Interestingly, our previous work indicates that oestradiol-treated ovariectomized rats demonstrate enhanced suppression of eating after acute amylin (Asarian *et al.*, 2011), suggesting that amylin sensitivity fluctuates over the course of the oestrous cycle, which could in part be the result of oestrogen-induced changes in RAMP expression.

We also provide evidence that RAMP2 mRNA is co-expressed with both RAMP1 and RAMP3 transcripts in over 50% of single AP cells; this highlights the possibility of co-expression of all three RAMPs along with CTRa in individual AP neurons. However, none of the individually collected AP neurons co-expressed CTRa and only RAMP2 mRNA; either one or both RAMP1 and RAMP3 transcripts were co-expressed with RAMP2 in single CTRa+ AP cells. These results further support the notion that amylin acts predominantly via $AMY_{1,3}$ in the AP, rather than AMY_2.

It is difficult to know at present whether these different AMYs have different physiological relevance; further studies are required to investigate the contribution of AP neurons carrying single vs. multiple RAMPs, in addition to the CTRa (i.e. CTRa/RAMP1, CTRa/RAMP3, CTRa/RAMP1/RAMP3 or CTRa/RAMP1/RAMP2/RAMP3 AMYs), to the binding and physiological effect of amylin. Our current work does not discern whether different variations of the AMY complex might contribute to the discrete physiological actions of amylin, such as the control of eating, increase in energy expenditure, slowing of gastric emptying or inhibition of glucagon secretion, all of which seem to be AP-mediated effects (Lutz, 2012; Hay *et al.*, 2015). Knockout or knockdown models of either one or more RAMPs would be an interesting experimental approach to answer such questions.

TABLE 1. CT values in single AP neurons following vehicle or amylin treatment

	Vehicle			Amylin		
Target	Average ± SEM	Minimum	Maximum	Average ± SEM	Minimum	Maximum
GAPDH	27.88 ± 0.55	26.88	34.96	27.30 ± 0.03	26.94	27.52
CTR	29.56 ± 0.59	28.14	37.30	29.13 ± 0.05	28.79	29.49
RAMP1	30.23 ± 0.44	28.96	36.79	32.03 ± 0.13	31.00	33.18
RAMP2*	33.32 ± 0.39	30.10	36.13	31.69 ± 0.95	24.80	37.98
RAMP3	26.79 ± 0.48	25.77	32.95	29.12 ± 0.05	28.65	29.68
LepRb	32.30 ± 0.73	30.40	37.01	27.58 ± 0.41	26.12	30.35

*RAMP2 was analysed in a separate experiment in which the GAPDH CT values were: vehicle, 31.74 ± 0.93 (minimum 24.27; maximum 36.82); amylin, 29.33 ± 1.00 (minimum 24.27; maximum 36.82).

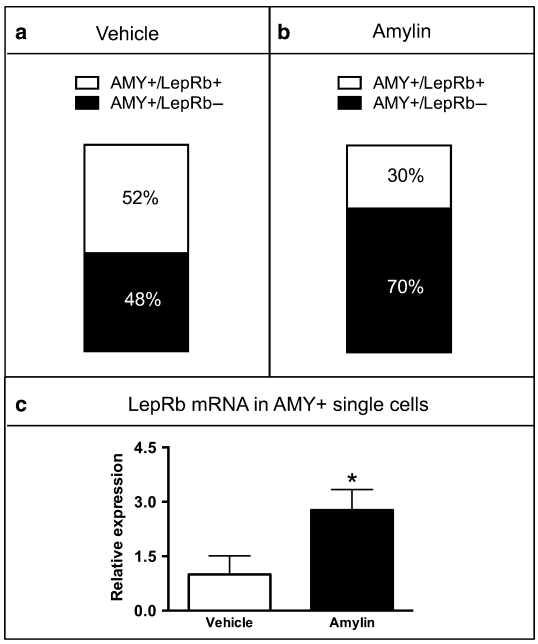

FIG. 6. Distribution of LepRb mRNA in single AP neurons bearing a functional AMY. Single cells (n = 4 rat/group; 9–10 cells/rat) that were positive for all components of the functional AMY (AMY+) were analysed for the presence or absence of LepRb mRNA. (a) In vehicle-treated rats, 52% of the single cells co-expressed AMY and LepRb mRNAs, whereas 48% of single neurons were found to be negative for the presence of LepRb. (b) In amylin-treated rats, 30% of the Fos+ single cells that were AMY+ also co-expressed LepRb mRNA. The remaining 70% of the cell population was described as AMY+ and LepRb−. (c) Quantitative analysis of LepRb mRNA levels; GAPDH was used as the housekeeping gene and mRNA expression is shown as fold change. LepRb transcript levels were significantly up-regulated in AMY+ cells from amylin-treated rats compared with vehicle-treated rats (*$P < 0.05$). Data shown as mean + SEM.

Our results also indicate that amylin has the potential to modify individual components of its own receptors. The relative quantification of the mRNA levels revealed that the expression of RAMPs but not of CTRa was differentially regulated by amylin. The mRNA of the primary contributors necessary to form the functional $AMY_{1,3}$ (RAMP1 and RAMP3) was consistently and significantly down-regulated by amylin, whereas CTRa mRNA seemed to be unaffected. In contrast to RAMP1 and RAMP3, RAMP2 mRNA was up-regulated after amylin administration. The functional consequences of the latter effect are unknown at present. The RAMP2 transcript level could be elevated to allow the dimerization with the calcitonin-like receptor in the rat AP, and therefore generate an adrenomedullin-responsive receptor over an AMY_2. However, this was not the purpose of our study.

The functional relevance of reduced RAMP1 and RAMP3 expression following amylin treatment is also currently unknown, but could be related to changes in amylin sensitivity. Interestingly, supporting evidence has shown that, when amylin was infused chronically at high doses (i.e. 50 μg/kg/day), its inhibitory effect on eating was stronger in the first few days of infusion compared with later time points (Lutz *et al.*, 2001), suggesting a decrease in amylin action. However, our recent work showed that, when rats are chronically treated with more physiological doses of amylin (5–10 μg/kg/day), the animals' responsiveness to acute amylin injections was maintained over time (Boyle *et al.*, 2011). Based on these data, we therefore hypothesize that amylin, especially when administered at supraphysiological concentrations, acts on its own primary receptors ($AMY_{1,3}$) to modulate RAMP1 and RAMP3 mRNAs via a potential negative feedback regulation mechanism. RAMP1 and RAMP3 may therefore act as the dynamic and regulatory component of the functional $AMY_{1,3}$ in single AP neurons, whereas CTRa may preferentially play a structural role as the core AMY subunit.

A number of studies have shown that amylin increases leptin sensitivity in leptin-resistant animals (Trevaskis *et al.*, 2010). Leptin exerts its eating and weight-lowering effects primarily by binding to LepRb. The LepRb-mediated effects on energy balance seem to rely on a wide network of brain areas, including the ventromedial nucleus and arcuate nucleus of the hypothalamus, but possibly also regions in the brainstem, such as the nucleus of the solitary tract, which is also rich in LepRb (Myers *et al.*, 2009). Although the ventromedial nucleus of the hypothalamus has been proposed to mediate the interactions between leptin and amylin (Trevaskis *et al.*, 2010), the involvement of the caudal brainstem, and specifically the AP, has not been fully examined. Interestingly, LepRb has also been found within the AP, although in low abundance (Wada *et al.*, 2014). Moreover, chronic amylin treatment in rats was shown to elevate both the basal and leptin-induced activation of phospho-signal transducer and activator of transcription 3 in the AP (Roth *et al.*, 2008), thus supporting the idea that amylin might also interact with leptin at the level of the AP (Trevaskis *et al.*, 2010). Here, we showed that, after acute amylin treatment, 30% of the single *Fos-GFP*+ AP cells co-expressed the transcripts of LepRb and AMY. Moreover, amylin up-regulated AP LepRb expression at the single-cell level. Our results therefore suggest the presence of a first-order neuronal population in the AP that is responsive to both amylin and leptin. This idea is consistent with recent findings that amylin and leptin are able to excite the same neurons isolated from the rat AP, using a whole-cell current-clamp recording technique (Smith *et al.*, 2015). Further, our observation that acute amylin treatment increased LepRb expression provides a possible mechanism by which amylin enhances leptin sensitivity, as was previously speculated (Trevaskis *et al.*, 2010). The specific contribution of AP neurons with LepRb to the effects of leptin on eating and to the interaction between amylin and leptin will require additional investigation.

The results from our current study provide the first concrete evidence that CTRa, RAMP1, RAMP2, RAMP3 and LepRb mRNAs are all co-expressed in single amylin-activated neurons of the native rat AP. Our data report transcriptional changes at the mRNA level only. This limitation was mainly due to the lack of availability of commercial antibodies against the RAMPs and LepRb; further studies would be important to show that the AMY components are also co-expressed at the protein level. Our data support the possibility of there being more than one AMY subtype in the same amylin-activated AP neuron. In addition, amylin has the potential to self-regulate its own receptors by modifying the transcriptional expression of the RAMPs. Finally, we showed that LepRb mRNA is co-expressed along with CTRa and at least one of the RAMPs in 30% of individual amylin-activated AP neurons. These data indicate that the AP may directly contribute to the interaction between amylin and leptin.

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Abbreviations

AMY, amylin receptor; AP, area postrema; CTR, calcitonin receptor; CTRa, calcitonin receptor isoform a; CTRb, calcitonin receptor isoform b; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; LCM, laser capture microdissection; LepRb, leptin receptor isoform b; RAMP, receptor activity-modifying protein.

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6. Original Research article: "The satiating hormone amylin induces neurogenesis in the *area postrema* of adult rats"

This section contains an original research article that was submitted for publication to the Journal of Cell Biology in February 2016.

My contribution to this manuscript includes the study design, data acquisition, data analysis, data interpretation and drafting and revising the manuscript.

Title Page

Title: The satiating hormone amylin induces neurogenesis in the *area postrema* of adult rats.

Running title: Amylin and adult neurogenesis

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Abstract

Adult neurogenesis in the subgranular zone and subventricular zone is generally accepted, but its existence in other brain areas is still controversial. Circumventricular organs, such as the *area postrema* (AP) have recently been described as potential neurogenic niches in the adult brain. The AP is the major site of action of the satiating hormone amylin. Amylin has also been shown to promote the formation of neuronal projections originating from the AP in neonatal rodents but the role of amylin in adult neurogenesis remains unknown. Here we performed an RNA-sequencing on the AP of adult rats and we showed that acute amylin affected genes involved in the control of adult neurogenesis (*e.g; NeuroD1*). Further, chronic amylin treatment (50 μg/kg/day) increased the number of newly proliferated cells. Using different cell markers, these adult-born cells were characterized as neurons. These results support a novel role of amylin in promoting neurogenesis in the AP of adult rats.

Significance Statement

Acute amylin treatment influences the transcription of many genes involved in neurogenesis-like processes (*i.e; NeuroD1*). Chronic administration of amylin strongly increases the number of newly proliferating BrdU-labeled cells in the AP of adult rats. The fate of the differentiated cells is mainly neuronal rather than glial. Our results show that amylin, next to its role as satiation signal, induces neurogenesis in the AP of adult rats.

Introduction

It is widely accepted that that the generation of new neurons and glial cells occurs continuously in adult mammalian brains. The subgranular zone (SGZ) of the hippocampus (Eriksson et al., 1998; Kempermann and Gage, 2000) and the subventricular zone (SVZ) lining the lateral ventricle (Doetsch et al., 1999) are two well-known brain regions where neurogenesis occurs during adulthood. The exact molecular mechanisms underlying adult neurogenesis are not yet fully understood. New-born cells in the SGZ and SVZ migrate to the dentate gyrus (DG) to develop into mature neurons, and to the olfactory bulb to became interneurons, respectively. Growing evidence supports the role of Ephrin-signaling to modulate the synaptic structure and physiological properties of neurons throughout their lifespan (Murai and Pasquale, 2004). In the adult brain, Ephrin receptors (EphRs) are expressed in the synaptic terminals where they influence synaptic plasticity and the commitment to neuronal fate (Murai and Pasquale, 2004). Moreover, the neurotransmitter gamma-amino-isobutyric acid (GABA) exerts similar actions in the adult brain by playing a key role in the control of multiple steps of adult neurogenesis. $GABA_A$-receptors are involved in synaptogenesis and plasticity in the adult brain and contribute to the proliferation, migration and differentiation of adult-born neurons (Fritschy and Panzanelli, 2014). Interestingly, in hippocampal slices, acute GABA application increased the expression of the transcription factor *NeuroD (neuronal differentiation)* within the SGZ (Tozuka et al., 2005). *NeuroD* is a positive regulator of neuronal differentiation in the terminal differentiation stage of the granule cells in the *dentate gyrus* (DG) (Tozuka et al., 2005). Finally, GABA-induced depolarization seems to be required for the integration of new neurons into the existing neuronal circuit, *e.g.* in the olfactory bulb (Belluzzi et al., 2003; Tozuka et al., 2005).

The occurrence of neurogenesis and gliogenesis has also been demonstrated in circumventricular organs (CVOs), including the area postrema (AP) (Bennett et al., 2009; Hourai and Miyata, 2013; Lin et al., 2015). The AP is a medullary structure located at the inferoposterior border of the fourth ventricle in the brainstem. The AP belongs to the family of sensory CVOs, which includes also the *organum vasculosum of the lamina terminalis* (OVLT) and the *subfornical organ* (SFO). CVOs are defined as "windows of the brain" because they possess permeable fenestrated capillaries and a highly deficient blood-brain-barrier (BBB) that allows neurons in the CVOs to directly receive information from the blood supply to control homeostatic functions (*i.e.,* pH balance, blood pressure, temperature, energy balance *via* hormones and metabolites, etc.) (Gross and Weindl, 1987; Joly et al., 2007; Roth et al., 2004). The lack of tight junctions in the CVOs, and specifically in the AP, might also allow access to important physiological stimuli that could further regulate neurogenesis in the adult brain (Lin et al., 2015).

The AP is a major brain site that mediates the effects of the satiating hormone amylin (Lutz, 2009; Lutz et al., 1998b; Riediger et al., 2001; Riediger et al., 2004). Amylin is co-secreted with insulin in response to nutrient stimuli (Lutz, 2010a) and mainly acts as a satiation signal to decrease food intake and body weight (Lutz et al., 1994; Trevaskis et al., 2010a). Amylin also inhibits glucagon secretion (Gedulin et al., 1997) and delays gastric empting (Young et al., 1995) *via* the AP. Although amylin-based research has primarily focused on its role in controlling food intake and body weight, a growing body of evidence suggests that amylin has additional effects on neuronal function. We previously showed that neonatal, amylin-deficient mice have reduced neuronal projections from the AP to the nucleus of the solitary tract (NTS), demonstrating that amylin has the potential to function as a trophic factor in the brain

(Lutz, 2010b), as had been shown earlier in the kidney, bone and pancreas (Lutz, 2010b; Wookey et al., 2006). It has also been shown that amylin may exert a neuroprotective effect in the brain. Chronic treatment with the amylin analog pramlintide improved memory and cognition, and ameliorated hippocampal pathology in a mouse model of Alzheimer's disease (Adler et al, 2014). Moreover, reduced neurogenesis in the hippocampus and the AP observed after ovariectomy in adult female rats was rescued by chronic infusion of amylin (Trevaskis et al, 2010). However, how amylin exerts the aforementioned effects is still unknown.

To investigate a possible role of amylin in enhancing neurogenesis in the AP of adult rats, we first used next generation sequencing (Ngs) to investigate the genes differentially affected by acute amylin treatment. Second, we examined whether chronic amylin infusion increased cell proliferation and differentiation in the AP of adult rats, using the BrdU-labeling and double fluorescence immunohistochemistry techniques.

Results

Acute amylin treatment affected pathways and Gene Ontology (GO) processes that participate in the regulation of neurogenesis.

To investigate the transcriptome of the entire AP of adult rats, animals (*n*= 5 *per* group) were acutely treated with vehicle, amylin (20 μg/kg) or with amylin and the amylin antagonist AC187 (500 μg/kg). cDNA libraries were generated from the extracted RNA and sequenced. Ngs data were processed with enrichment analysis for biological pathways and GO processes. Pathways analysis revealed that Cell adhesion_Ephrin signaling, neurophysiological process_$GABA_A$-receptor life cycle and neurophysiological process_ $GABA_B$-receptor signaling at postsynaptic sides of the synapsis pathways were the top-three pathways which involved genes affected by acute amylin treatment (Table 1).

GO analysis described a scenario in which most of the genes that were affected by amylin in the AP are involved in neurogenesis-like processes, in particular in synaptic transmission, nervous system development, cell-cell signaling and neurogenesis (Table 2). A cross analysis between the major pathways and GO processes revealed the *NeuroD (Neuronal-differentiation)* gene family as the main group of genes involved in the aforementioned processes. Network analysis defined a major gene map in which members of the Ephrin-receptor signaling pathway, the $GABA_A$-receptor signaling pathway, and genes involved in the regulation of neurogenesis (*i.e., NeuroD1, WNT*) all constitute an effective gene network affected by acute amylin in the AP of adult rats (Figure 1).

Acute amylin administration significantly upregulated NeuroD1 transcript and protein in the AP of adult rats, and this response was completely blocked by the administration of AC187.

NeuroD1 is a member of the *NeuroD* gene family which is required in the final step of the neural stem cell differentiation to acquire the neuronal cell fate (Gao et al., 2009; Pataskar et al., 2015). Results from the GO enrichment analysis (Table 2) showed that the majority of the genes identified *via* the Ngs are involved in neurogenesis-like processes; further, the gene network (Figure 1) described a scenario in which *NeuroD1* may interact, *via* canonical and unknown pathways, with EphRs and $GABA_A$-receptors signaling to mediate amylin's effect on neurogenesis-like processes.

We further validated the effect of amylin on *NeuroD1* mRNA expression by qPCR and found that acute amylin treatment significantly upregulated *NeuroD1* transcript levels about 13 fold, and that this effect was completely reversed by AC187 (Figure 2a; One-way ANOVA; $F_{(2,12)} = 18.96$, $^{***}P = 0.0002$). We also confirmed the upregulation at the protein level and showed that rats (*n=3 per group*) that were acutely treated with amylin (20 μg/kg; *i.p.*) exhibited a marked upregulation of NeuroD1 (Figure 2b-d; Student t-test; $t_4=9.774$, $^{***}P= 0.0006$ [Figure 2b]). The number of positive cells (*i.e;* number of particles) was quantified with Image J version 1.49b.

Chronic amylin treatment significantly increased the number of newly proliferating (BrdU-labeled) cells after one-week treatment in the AP of adult rats.

To further investigate the role of amylin in adult neurogenesis, animals (*n*=8 *per* group) were chronically treated with amylin (50 µg/kg/d) or vehicle (Figure 3 a,b). Based on the premise that new cells are continuously generated in the AP of adult rats (Bennett et al., 2009; Hourai and Miyata, 2013), we examined the number of proliferating cells by analyzing BrdU-immunoreactive (BrdU-labeled) cells. After one-week of amylin infusion, the number of BrdU-labeled cells was significantly increased compared to controls (Student t-test; t_{12}= 3.978, **P= 0.0018; Figure 3c). As a positive control for an effect of amylin, food intake was measured daily throughout the experiment; as expected (Lutz et al., 2001), amylin effectively decreased 24-hours food intake (but not body weight) compared to controls (Student t-test; t_{14}= 2.574, *P < 0.05; Supplementary Figure 1a,b).

BrdU-labeled cells mainly differentiated into neurons after three-weeks of chronic amylin treatment in the AP of adult rats.

To determine the cell fate of the newly born cells in adult rats, we performed a series of immunohistological double-labeling stainings using different cellular markers after three weeks of amylin treatment (Figure 4). Immature and mature neurons were stained with the neuronal marker HuCD (Figure 4; b,e) and $BrdU^{+}/HuCD^{+}$ double-labeled cells were mainly present in the central region of the AP (Figure 4f). Glial cells were identified with the glial marker GFAP (Figure 4h,k) and $BrdU^{+}/GFAP^{+}$ double-labeled cells were predominantly located at the borders of the AP (Figure 4i,l). The number of BrdU-labeled cells was higher in rats chronically treated with amylin

compared to controls (Student t-test; t_{15}=2.163, *P=0.0471; Figure 5a). Moreover, amylin significantly increased the number of $BrdU^{+}/HuCD^{+}$ double-labeled cells, compared to control (Student t-test; t_{15}=6.059, *P < 0.0001; Figure 5b). Finally amylin significantly raised the percentage of double-labeled cells in respect to the percentage of BrdU-positive cells (Student t-test; t_{15}=5.563, *P < 0.0001; Figure 5c). Confocal quantification confirmed the double-staining observed under fluorescence microscope (Figure 6a) and showed that amylin preferentially induced neuronal rather than glial cell fate (One-way ANOVA; $F_{(3,28)}$= 27.51; ****P < 0.0001; Figure 6b).

To confirm a chronic amylin effect, food intake and body weigh were monitored daily during the experiment. 24-hours food intake (but not body weight) was significantly decreased by amylin during the three-week treatment period (Student t-test; t_{14}=2.718, *P=0.0167; Supplementary Figure 1c,d).

Finally, to exclude a possible co-localization of BrdU-labeled cells with interneurons or blood vessels, we performed immunohistochemical analysis for the interneuronal marker claretitinin (CR) (Supplementary Figure 2 a-f) and the blood-vessel marker RECA-1, respectively (Supplementary Figure 2 g-m). We did not detect any co-localization of BrdU-labeled cells with CR (Supplementary Figure 2f), indicating that chronic amylin does not seem to promote the differentiation of newborn cells into interneurons in the AP. Abundant RECA-1 staining was found in the AP, confirming its highly vascularized structure (Supplementary Figure 2h,l); however, no co-localization between BrdU and RECA-1 was detected in the AP of adult rats (Supplementary Figure 2i,m).

Discussion

We demonstrate that acute amylin treatment affected the regulation of genes that are involved in neurogenesis-like processes. In particular, the Ngs data analysis showed that acute amylin treatment consistently affected Ephrin-signaling and $GABA_A$-signaling pathways. Moreover, GO processes revealed that acute amylin affected genes mainly involved in synaptic transmission, nervous system development, cell-cell signaling and generation of new neurons. *NeuroD1* was identified as the major gene up-regulated by acute amylin administration. This effect was reversed by the administration of the amylin receptor antagonist AC187, and immunohistochemistry confirmed the increase in *NeuroD1* expression by amylin also at the protein level. These findings are consistent with previous evidence of the involvement of EphR signaling, $GABA_A$-receptors signaling and NeuroD-gene family signaling, in the regulation of different steps during adult neurogenesis (Fritschy and Panzanelli, 2014; Murai and Pasquale, 2004; Tozuka et al., 2005).

Moreover, our BrdU study confirmed previous findings that CVOs, including the AP of adult rats, contain constitutively proliferating cells that go on to differentiate into neurons and glia (Bennett et al., 2009; Lee et al., 2012). Therefore, CVOs might be considered as new neurogenic niches (Lin et al., 2015), next to their role in mediating homeostatic controls in the adult brain. *In vivo*, we observed a clear effect of chronic amylin treatment on the AP of adult rats and we report that amylin not only increased cell proliferation, but also promoted the commitment to neuronal fate of adult-born AP-cells. In fact, confocal microscopy analysis demonstrated that the majority of newly proliferated cells in the AP of adult rats differentiated into neurons. Only a small percentage of adult-born cells, mainly located at the edges of the AP,

differentiated into glia three weeks after BrdU labeling.

At present, the functional significance of continuous neurogenesis in the adult AP is not well understood. A potential explanation could be that the generation of new neurons is required to control homeostasis. Specifically, the ongoing neurogenesis might be responsible for a fine control of food intake and vomiting (Lutz, 2006; Miller and Leslie, 1994), two main actions controlled by the AP by a large number of physiological or pathological stimuli. Hence, the necessity for the constitutive neurogenesis in the adult AP might resides in the "CVO-nature" of the AP. The lack of a BBB, and the possible negative influence on AP neurons due to blood-derived substances might affect the efficiency and survival of the AP-neurons. Therefore, the presence of new neurons in this brain area might suggest a protective mechanism of neuronal repair. Interestingly, other brain areas involved in nutrient sensing and in the control of eating have also been reported to shown adult neurogenesis. In the median eminence, tanycytes from the ependymal zone generate new neurons that contribute to the control of food intake (Lee et al., 2012). In the arcuate nucleus, the ciliary neurotrophic factor mediates continuous neurogenesis which has been shown to affect the long-term regulation of energy balance (Kokoeva et al., 2005).

Recent work from our group showed that neonatal, amylin-deficient mice have reduced neuronal projections from the AP to the NTS, demonstrating that amylin has the potential to function as a trophic factor in the brain of newborn rats (Lutz, 2010b). Moreover, chronic amylin administration was shown to compensate for the reduction in neurogenesis in the hippocampus and AP observed in adult female ovariectomized rats (Trevaskis et al, 2010). Finally, it was also shown that chronic treatment with pramlintide, an amylin analogue, improved memory and cognition and ameliorated

hippocampal pathology in a mouse model of Alzheimer's disease (Adler et al, 2014). These findings suggest that amylin-induced neurogenesis in adult rats may play a previously unappreciated role in physiology and disease. How amylin exerts the aforementioned effects is unknown at present. A potential mechanism might involve amylin-signaling *via* the extracellular signal-regulated kinase 1 and 2 (ERK 1/2) cascade. It has been shown that ERK1/2 knockout mice have impaired neurogenesis, suggesting that ERK1/2 activity is required for the generation of new neurons (Satoh et al., 2011). Further, our own studies showed that amylin time and dose-dependently activates ERK 1/2 signaling, by inducing ERK phosphorylation. Since pERK-positive AP-neurons partially overlap with the core subunit of the amylin receptor, calcitonin-receptor (CTR) (Potes et al., 2012), this effect seems to be mediated by CTR-signaling. These findings suggest that amylin may influence AP-adult neurogenesis *via* ERK phosphorylation.

It also remains to be addressed how the adult-born AP-neurons are functionally integrated into pre-existing circuits and how amylin influences this process. Future work is needed to determine whether amylin also enhances neurogenesis in the AP of newborn rats.

To confirm the presence of an amylin effect, food intake and body weight were monitored daily throughout the course of the chronic experiment. Food intake was significantly reduced in amylin-treated rat compared to control. Unexpectedly, amylin did not exert a lasting effect on body weight. As weight loss *per se* is known to induce neurogenesis in the hippocampus (Mattson, 2000), we can therefore exclude an effect of decreased body weight as a source of increased neurogenesis in the AP of adult rats.

In summary, our results indicate that amylin increases the production of new neurons in the AP of adult rats. Analyses of biological pathways, GO processes and gene network suggest that acute amylin differentially regulates genes involved in neurogenesis-like processes *in vivo*. A potential mechanism for this phenomenon may relate to an activation of a complex cellular network by amylin, possibly involving ERK1/2 signaling and operating *via* EphRs and $GABA_A$-receptor pathways. This might lead to the activation of *NeuroD1*, which acts as the necessary signal to commit adult-born AP-cell to the neuronal fate. Moreover, chronic amylin treatment increased both cell proliferation and cell differentiation into neurons. These results support a novel role of amylin in the processes of adult AP neurogenesis, providing new insights into the mechanism of neurogenic regulation.

Materials and methods

Animals and housing

Adult male Wistar rats (Janvier, Le Genest Saint Isle, France) (220-250 g) were used for the experiments and were single-housed in a temperature-controlled environment (21±1°C) on an artificial 12h/12h light/dark cycle. Rats had *ad libitum* access to water and standard chow, except during fasting periods as described below. All procedures involving animals and their care were approved by the Veterinary Office of the Canton Zurich, Switzerland, and in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

Drugs

Amylin (Bachem AG, Bubendorf, Switzerland; catalogue number: H-9475.1000) was reconstituted with sterile 0.9% NaCl and AC187 (TOCRIS Bioscience, Bristol, UK; catalogue number: 3419) was reconstituted in sterile double-distillated water. 5'-bromo-2-deoxyuridine (BrdU, B5002, Sigma Aldrich, Buchs, Switzerland) was dissolved in sterile double-distillated water and heated to 40-50°C.

Experimental design

Experiment 1

For the Ngs experiment, rats (220-250 g; *n*=5 *per* group) were fasted during the light phase. At dark onset, rats were acutely treated *i.p.* with vehicle (NaCl), amylin (20 µg/kg), or with amylin plus the amylin receptor antagonist, AC187 (500 µg/kg). Ninety minutes after drug administration, rats were anesthetized with isoflurane and decapitated; during this 90-min period, rats had no access to food.

RNA extraction

After decapitation, the AP was surgically removed from the hindbrain under a light microscope and RNA was extracted according to manufacturer's instructions (TRI Reagent®; Sigma-Aldrich, Buchs, Switzerland) and then purified following the cleanup protocol of RNeasy® Mini kit (Qiagen, Basel, Switzerland), including the DNase step. The concentration and the integrity of RNA were measured using a nanodrop system (NanoDrop 1000 Spectrophotometer, (Thermo Scientific, Waltham, MA, USA).

Strand-specific cDNA library construction

From each sample, 10 ng of total RNA were taken to construct strand-specific cDNA libraries using the Ovation® Single Cell RNA-Seq System (PART NO. 0342, NuGEN; San Carlos, California). The quality of cDNA libraries was tested and quantified cDNA was subjected to purification, elution, end-repair and adaptor ligation. A second round of library amplification and purification was performed, following the manufacturer's instruction. Samples were screened by bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and qPCR. The established cDNA libraries were sequenced using the Illumina HiSeq 2000 platform (Ilumina Inc., San Diego, CA, USA) to generate 100bp paired-end reads.

Quantitative real-time PCR (qPCR)

Total RNA was reverse transcribed with Tetro cDNA Synthesis Kit (Bioline, Elchrom Scientific AG, Cham, Switzerland). Real-time PCR was performed using 7500 Fast system (Applied Biosystem/Life Technologies Carlsbad, CA, USA) with QuantiTect® SYBR® green PCR kit (Qiagen, Basel, Switzerland). Intron-spanning

primers were designed with IDT, Integrated DNA Technologies, (http://eu.idtdna.com). The primer sequences were the following: for rat GAPDH, forward: 5'-AGACAGCCGCATCTTCTTGT-3' and reverse: 5'-CTTGCCGTGCGTAGAGTCAT -3' (Accession: NM_017008.4); for rat NeuroD1, forward: 5'-GAACACGAGGCAGACAAGAA-3' and reverse: 5'-TCATCTTCATCCTCCTCCTCTC-3' (Accession: NM_019218.2). 100 ng of cDNA were subjected to an initial heat activation at 95 °C for 15 minutes, followed by 40 cycles of alternating between 94 °C for 15 sec, 60 °C for 30 sec, and final extension at 72°C for 30 sec. The fold change in expression of each gene was calculated using the comparative ΔΔCt method, with GAPDH as endogenous control. Each sample was run in duplicate.

Enrichment analysis for pathways, GO processes and gene network construction

The list of ranked genes for each sequencing batch and the list of susceptibility genes were imported into MetaCore™ software version 6.24 (Thomson Reuters, New York, NY, USA). Enrichment analysis for pathways and GO processes was performed on gene expression data to identify significant pathways and processes affected by our experimental conditions. Enrichment analysis comprised both up- and down- signals, distribution was achieved by intersection setting and the sorting method was based on statistically significant data (threshold=2 and p-value $\leq$ 0.01). Analysis was performed *via* the online platform portal.genego.com version 6.20.66481. To determine the pathways associated with the susceptibility genes, a sub-enrichment analysis was performed on the list of these genes. Pathways are represented by the GO set class of biological processes. Finally, to determine the interactions between susceptibility genes from the different functional groups, gene networks were

constructed. Data files were analyzed with MetaCore+MetaDrug® version 6.19 build 65960 scripts 65979.

Tissue preparation

Animals were deeply anesthetized with an *i.p.* injection of sodium pentobarbital (100 mg/kg) and transcardially perfused using 0.1M PBS followed by 4% paraformaldehyde (PFA).The brains were collected and maintained overnight in 4% PFA. Subsequently, the brains were transferred to 20% sucrose in PBS for 24 hours. Brains were cut into 20-µm coronal sections on a cryostat (Leica microsystem, Wetzlar, Germany) and stored in cryoprotectant (20% glycerol, 30% ethylene glycol, 50% 0.1M PB) until processing for immunohistochemistry.

Immunohistochemistry

Slide-mounted sections (20 µm) were washed in 0.1M PBS (pH 7.4) and then incubated in immunoblocking buffer (2.5% bovine serum albumin [BSA], 0.1% Triton X-100 in PBS) for 1 h at room temperature. Subsequently, sections were incubated with Rabbit anti-NeuroD1 primary antibody (1:250, Abcam, Cambridge, UK). After overnight incubation at 4°C, the sections were washed in PBS and incubated for 3 h with fluorescent secondary antibody Alexa Fluor 488 Donkey anti-rabbit immunoglobulin G (IgG) (1:400, Invitrogen, MOLECULAR PROBES®, Eugene, Oregon, USA). The nuclear dye DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich, Buchs, Switzerland) was added to secondary antibodies solution for nuclear staining. Sections were then rinsed in PBS and coverslipped with fluorescent mounting medium.

Experiment 2

Rats (220-250 g; 4 groups and *n*=8 *per* group) were acclimatized in their home-cage environment. Amylin (50 μg/kg/d) or vehicle was chronically administered peripherally by subcutaneous osmotic minipumps (Alzet®, Model 2002; Durect Corporation; Cupertino, CA, USA) for seven days or three weeks, respectively. On the day of implantation, the minipumps were filled under sterile conditions with saline or amylin. Rats were initially anesthetized by inhalation of 5% isoflurane (IsoFlo®, Provet AG, Lyssach, Switzerland), then maintained on 2-3% isoflurane and placed on a heating pad to maintain body temperature during surgery. At the site of implantation, rats were shaved and the skin was disinfected with Betadine® (Provet AG, Lyssach, Switzerland). Under sterile conditions, a small incision was made between the scapulae and the minipump was subcutaneously implanted. The wound was closed with interrupted cutaneous sutures. Food intake and body weight were measured daily.

BrdU injection

BrdU was used to label proliferating cells and to track the fate of newly proliferated cells. Rats were given twice daily intra-peritoneal (*i.p*) injections of BrdU (75 μg/kg; 12 h apart) for 6 days, starting on the day of amylin treatment. To determine the rate of newborn cells, part of the rats were sacrificed on day 7, i.e. 12h after the last BrdU injection; the remaining rats were allowed to survive for an additional two weeks and then sacrificed at the end of the 3-week amylin infusion period, to investigate the fate of the BrdU-labeled cells.

Tissue preparation

Animals were treated following the protocol described in *Experiment 1*, with two exceptions: here, tissues were stored in a solution of 30% sucrose in PBS for 48 hours and brains were cut into 30-μm coronal sections.

Double fluorescence Immunohistochemistry

Free-floating sections were pretreated to denature the DNA to make the BrdU antibody detectable. Briefly, AP sections were incubated in a solution of 50% formamide for 2h at 65°C; then, sections were incubated in 2N HCl for 30 min at 30°C and subsequently acid neutralized with 0.1 M borate buffer for 10 min. After washing in 0.1M PBS (pH 7.4), sections were incubated in immunoblocking buffer (3% goat serum [NGS], 1% bovine serum albumin [BSA], 0.3% Triton X-100 in PBS) for 1 h. Primary antibodies were applied as a cocktail that included rat anti-BrdU primary antibody (1:100, Abcam, Cambridge, UK), mouse anti-HuC/D (1:100, Thermo Fisher Scientific, Wahltman, MA, USA), rabbit anti-DCX (1:1000, Abcam, Cambridge, UK), rabbit anti-GFAP (1:1000, Abcam, Cambridge, UK), mouse anti-RECA-1 (1:2000; AbD Serotec, Kidlington, UK) or rabbit anti-calretinin (CR) (1:100, Abcam, Cambridge, UK). After overnight incubation at 4°C, the sections were washed in PBS and incubated for 4 h with fluorescent secondary antibodies, also applied as a cocktail: Alexa Fluor 488 goat anti-rat immunoglobulin G (IgG) to reveal immunoreactivity of BrdU and Alexa Fluor 555 goat anti-rabbit to reveal immunoreactivity of GFAP, RECA, DCX and CR IgG, respectively (1:400 for all the antibodies; Invitrogen, MOLECULAR PROBES®, Eugene, Oregon, USA), or Alexa 555 goat anti-mouse to reveal immunoreactivity of HuCD. Then, the sections were

rinsed in PBS and mounted onto Superfrost glass slides (Thermo Scientific, Waltham, MA USA) and coverslipped with fluorescent mounting medium.

Cell quantification and Analysis

Two AP sections per brain were counted and averaged to determine the number of BrdU-labeled cells. Counting was accomplished at 20 X magnification and numerical aperture of 0.5, using a light microscope (Zeiss Imager Z2; Carl Zeiss, Jena, Germany) equipped with a color camera (Zeiss Axiocam; Carl Zeiss, Jena, Germany). For cell differentiation experiments, fluorescent double-labeled sections with $BrdU^+/HuCD^+$ cells and $BrdU^+/GFAP^+$ cells were visualized and analyzed on CLSM Leica TCS SP8 upright confocal microscope (Leica Microsystem, Wetzlar ,Germany) equipped with an *x-y-z* motorized stage and three lasers (Argon 488, HeNe 543 and HeNe 633) appropriate to detect DAPI, Alexa 555 and Alexa 488 fluorescence, respectively. Each laser channel was separately scanned using a multitrack PMT configuration to avoid cross-talk between fluorescent labels. A careful examination of all secondary antibodies was performed to avoid any possible cross-talk between fluorescent dyes or any cross-reactivity between secondary antibodies. To evaluate double-labeling, confocal *z*-stack sectioning was performed at 0.5-1.0-µm intervals using 40 X oil-immersion (NA= 1.30) or 63 X oil-immersion (NA=1.40) objectives. Images were acquired and 3D-reconstructed using the Zeiss LSM software, cropped and optimized in Imaris Software version 6.4.0 (Bitplane AG, Zurich, Switzerland). Colocalization of BrdU with HuCD or GFAP, respectively, was confirmed by examining multiple optical planes for each cell on the *z*-axis. The percentage of BrdU-positive cells double-labeled for HuCD or GFAP was determined by eye counting. The experimenter was blinded to the treatment (amylin *versus* control).

Supplemental material

Suppl. Figure 1 shows 24 hours-food intake and body weight of rats after one and three-weeks of amylin treatment, respectively. Suppl. Figure 2 shows lack of co-localization of BrdU with the interneuron marker CR and of BrdU and the blood vessel marker RECA-1.

Statistical analysis

Relative mRNA expression levels were assessed by one-way ANOVA. BrdU-labeled cells and food intake were quantified and then compared using Student's t-test or one-way-ANOVA, as appropriate, by using GraphPad Software version 6.0 (San Diego, CA, USA).

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Abbreviation list

AP, *area postrema*; BrdU, 5'-bromo-2-deoxyuridine; BBB, blood-brain barrier; CNS, central nervous system; CVO, circumventricular organ; CR, Calretinin; CTR; calcitonin receptor; DG, dentate gyrus: EphRs, Ephrin receptors; ME, median eminence; *GABA-A Receptor, Gamma-Aminobutyric Acid A Receptor*); GFAP, glial fibrillary acidic protein; GO, gene onthology; *NeuroD*; *neuronal differentiation*; *NeuroD1*, *neuronal differentiation-1*; Ngs, next-generation-sequencing; NTS, *nucleus tractus solitarius*; OVLT, *organum vasculosum of the lamina terminalis*; SFO, *subfornical organ:* SGZ*, subgranular zone:* SVZ*, subventricular zone.*

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Figures and Figure legend

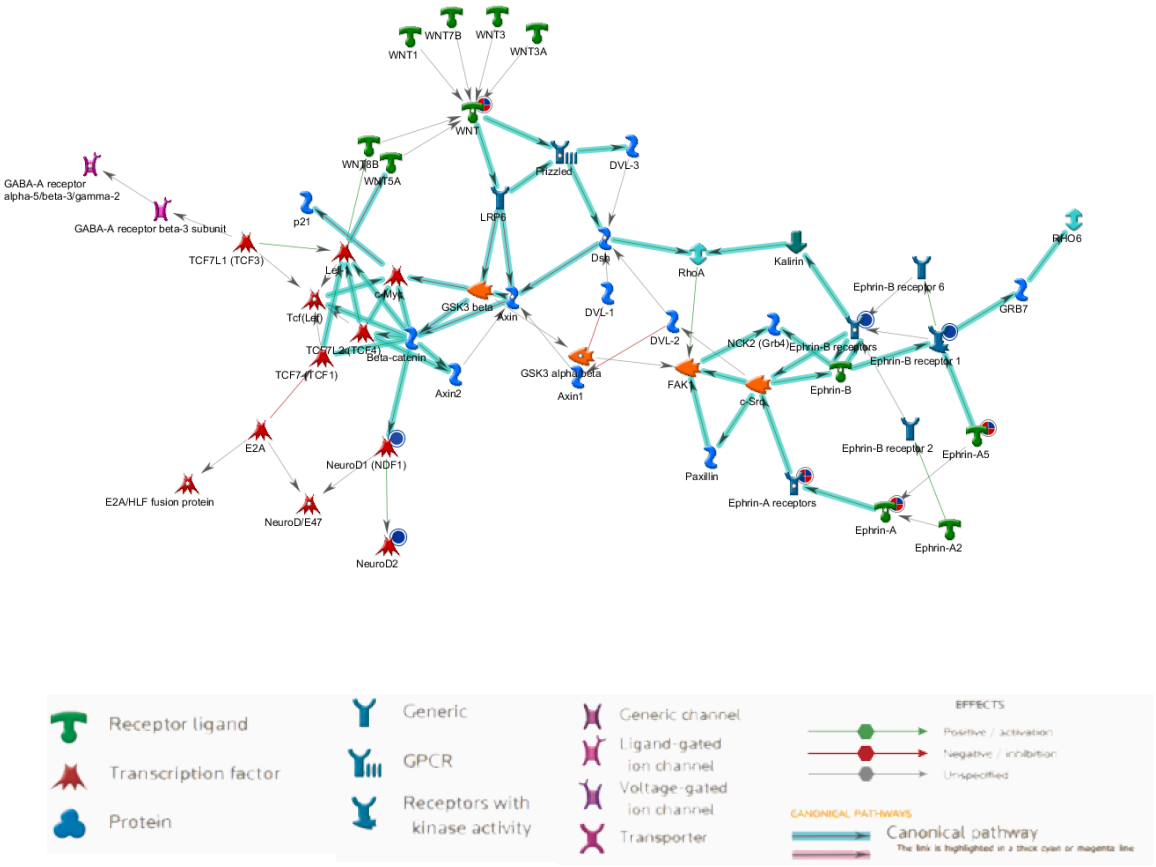


Figure 1

Top gene network in response to acute amylin. Network analysis (generated with MetaCore+MetaDrug®) of the genes whose expression was affected by amylin treatment are shown. Major functions of the network are indicated. See the legend for details.

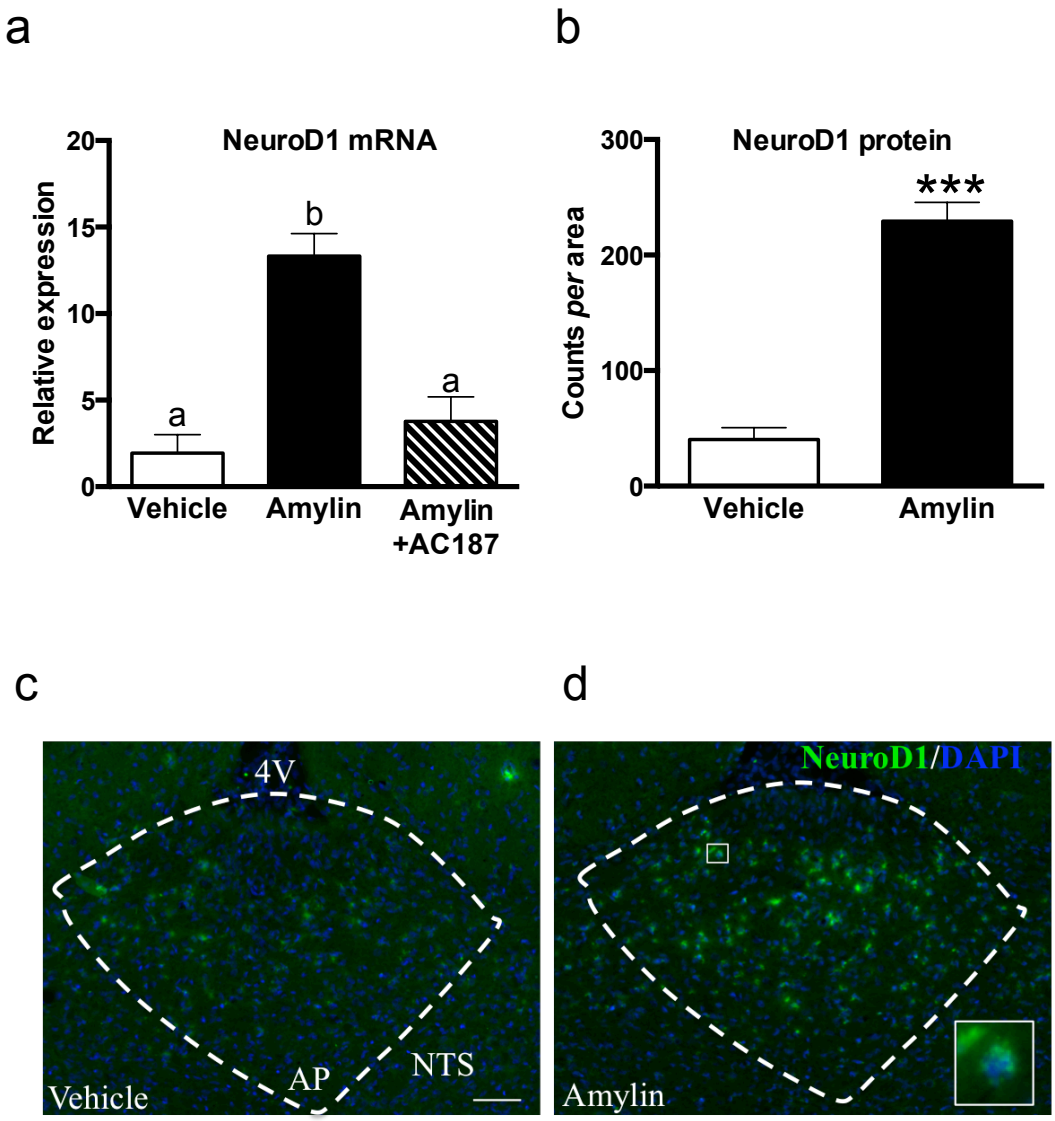


Figure 2

NeuroD1 mRNA and protein expression was significantly increased by acute amylin treatment. (a) qPCR analysis showing *NeuroD1* mRNA expression levels in vehicle, amylin (20 μg/kg) and amylin+AC187 (500 μg/kg) treatments (*n*=5 rats *per* group). Amylin consistently and significantly up-regulated *NeuroD1* mRNA expression compared to vehicle. AC187 reversed the amylin effect (One-way ANOVA, ***P=0.002). mRNA levels are shown as fold change and GAPDH is used as internal control. Data are expressed as mean ± S.E.M. (b) Quantification of NeuroD1 protein. (c) Immunohistochemistry of NeuroD1 in AP sections of a vehicle (c) and amylin (d) treated rat, respectively. The inset shows co-localization of NeuroD1 and DAPI. Scale bar represents 50 μm.

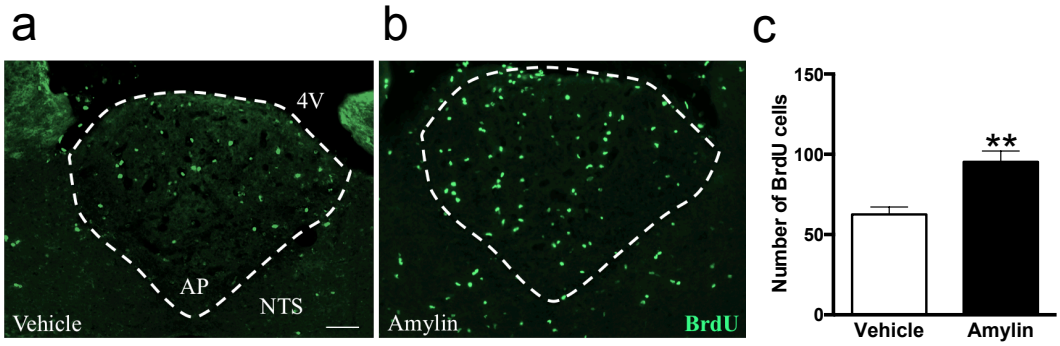


Figure 3

Amylin (50 µg/kg) increased the number of BrdU-positive cells after one week of treatment. (a) Immunohistochemical staining of BrdU-positive cells in AP sections of vehicle (a) and amylin (b) treated rats, respectively (*n*=8 rats *per* group). (c) Amylin significantly increased the number of BrdU-immunoreactive cells compared to control (Student t-test; $^{**}P = 0.0018$). Data are expressed as mean ± S.E.M. Scale bar represents 50 µm.

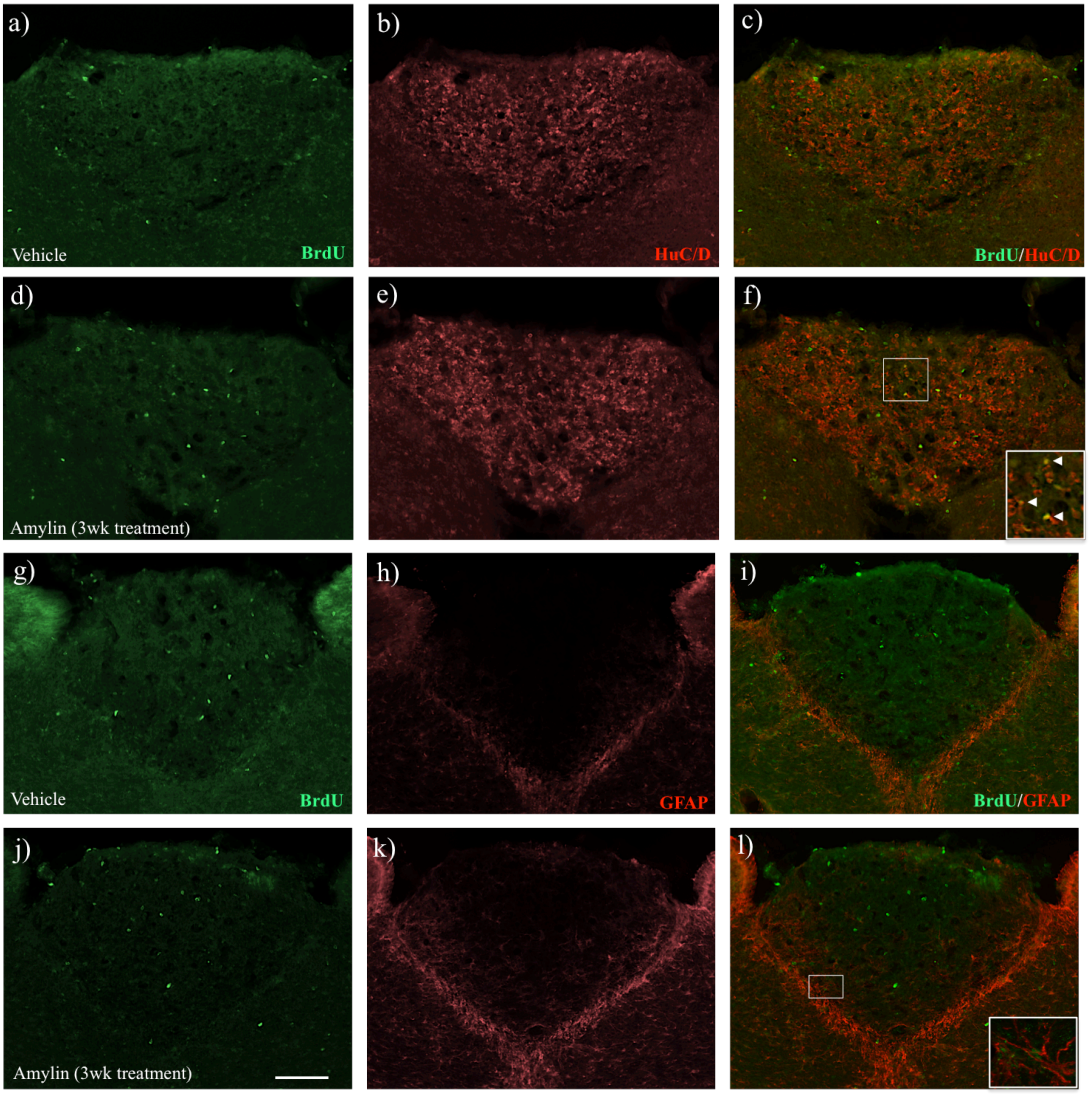


Figure 4

BrdU-labeled cells in the AP of vehicle (a,g) and amylin (d,j) treated rats (n = 8 per group); amylin was given for 3 weeks at a dose of 50 µg/kg/day. Sections stained for neuronal (HuCD) and glia (GFAP) markers, respectively, are shown in (b,e,h,k). Newly generated BrdU-positive neurons double labelled for HuCD and GFAP are shown in (c,f) and (i,l), respectively. The insets show examples of colocalization of BrdU and HuCD (f), which represent neurons, or colocalization of BrdU and GFAP (l), which represent glial cells. Scale bar represents 50 µm.

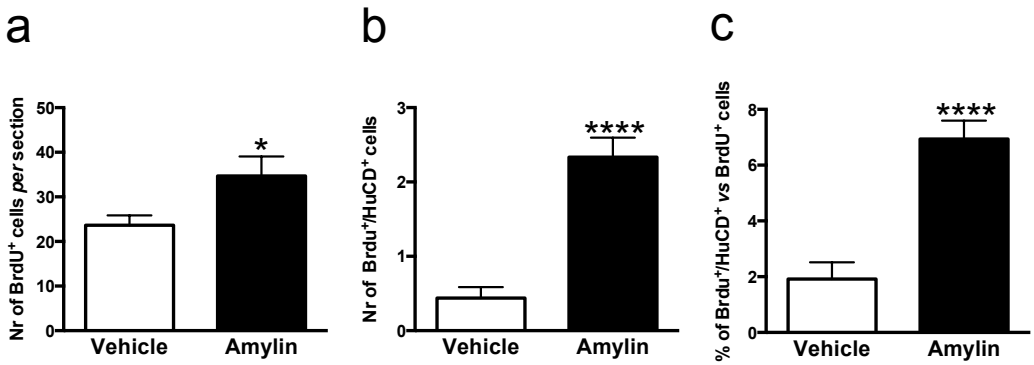


Figure 5

Quantitative analysis of $BrdU^+$ and double-labeled AP cells after 3 weeks of vehicle or amylin (50 μg/kg) treatment (*n*=8 rats *per* group). (a) Amylin significantly increased the number of BrdU-labeled cells after 3 weeks of treatment (t-test; $^{*}P < 0.05$). (b) The number of double labelled cells ($BrdU^+/HuCD^+$) cells was increased after amylin treatment, compared to control (t-test; $^{****}P < 0.001$). (c) The percentage of double-labelled $BrdU^+/HuCD^+$ cells in respect to the total number of $BrdU^+$ cells (t-test; $^{****}P < 0.001$). Data are expressed as mean ± S.E.M.

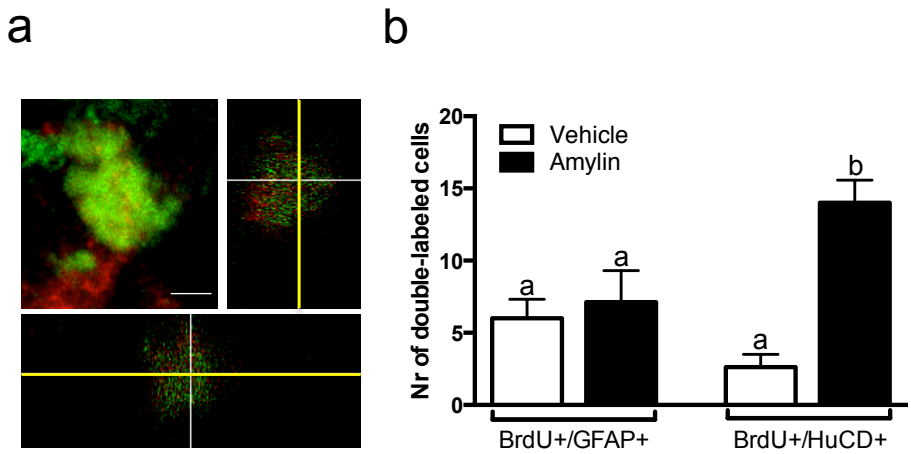


Figure 6

Quantification of double-labeled cells. (a) Confocal *z*-stack image showing the co-localization of $BrdU^+$ and $HuCD^+$ cells. Scale bar represents 5 µm. (b) Number of double-labeled $BrdU^+/GFAP^+$ and $BrdU^+/HuCD^+$ cells in the AP of adult rats. Adult-born cells preferentially committed to neuronal rather than glial fate after chronic amylin (50 µg/kg/d) treatment (One-way ANOVA, b= ****P<0.0001). Data are expressed as mean ± S.E.M.

Tables

Table 1. Enrichment analysis by pathway

Pathway Maps	*Total	p-value	FDR
Cell adhesion_Ephrin signaling	45	1.384E-07	2.076E-05
Neurophysiological process_GABA-A receptor life cycle	27	4.311E-06	7.674E-04
Neurophysiological process_GABA-B receptor signaling at postsynaptic sides of synapses	42	2.628E-05	2.339E-03
Neurophysiological process_Dopamine D2 receptor signaling in CNS	47	4.125E-05	2.448E-03
Signal transduction_PKA signaling	51	5.711E-05	2.541E-03
Neurophysiological process_Receptor-mediated axon growth repulsion	45	1.010E-04	7.573E-03
Neurophysiological process_Dopamine D2 receptor transactivation of PDGFR in CNS	26	1.530E-04	1.683E-02
Neurophysiological process_HTR1A receptor signaling in neuronal cells	26	1.530E-04	1.683E-02
G-protein signaling_G-Protein alpha-i signaling cascades	27	1.856E-04	6.608E-03
Neurophysiological process_Mu-type opioid receptor-mediated analgesia	30	2.554E-04	7.578E-03

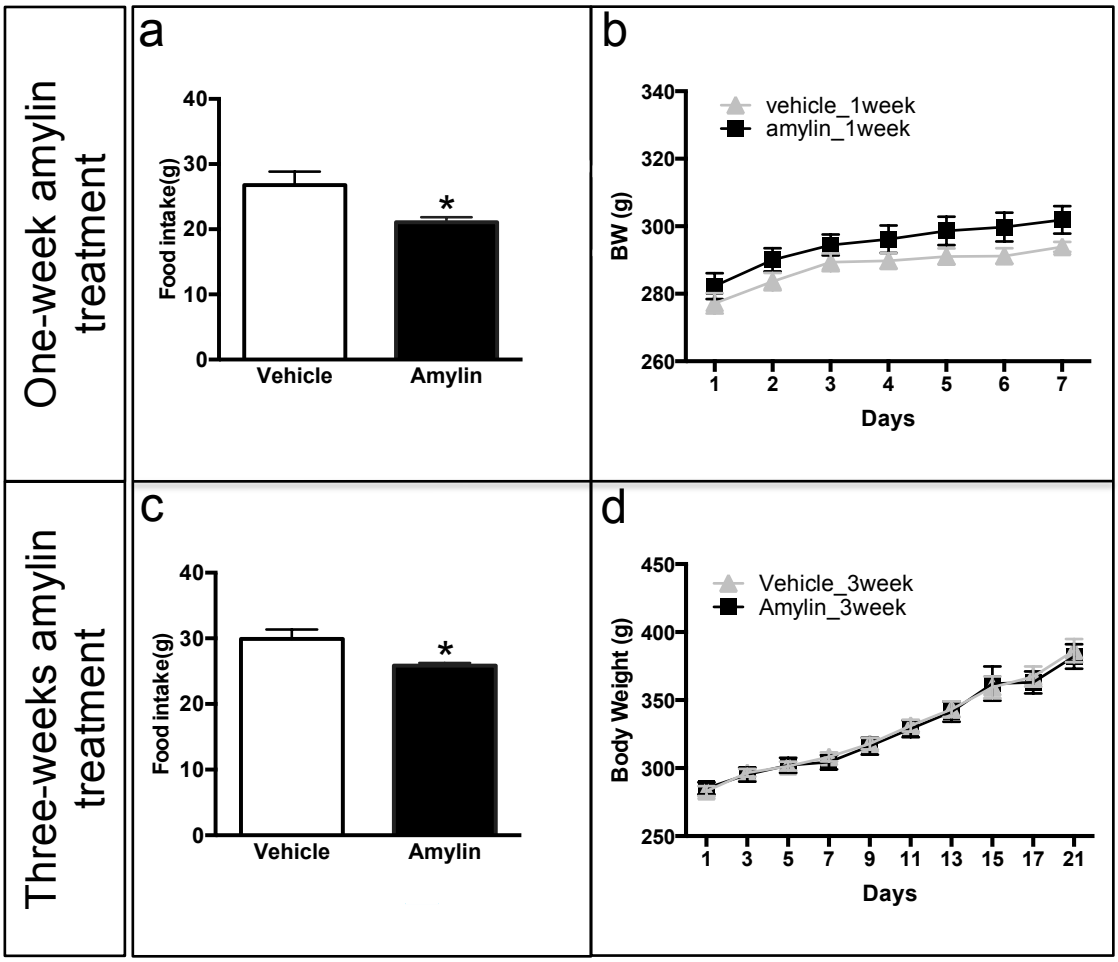
*Total refers to the total number of genes involved in a pathway. FRD and p-value refer to pathways involved in the experimental conditions. A p-value < 0.01 is considered significant.

Table 2. Enrichment analysis by GO processes

Processes	*Total	p-value	FDR
Synaptic transmission	874	6.897E-33	2.664E-29
Nervous system development	2841	4.102E-30	7.922E-27
Cell-cell signaling	1293	6.233E-28	9.147E-25
Neurogenesis	1969	5.902E-21	5.698E-18
Neurotransmitter transport	185	1.526E-20	1.179E-17
System development	5219	2.760E-20	1.776E-17
Neurotransmitter secretion	133	3.607E-20	1.990E-17
Regulation of neurotransmitter levels	190	4.640E-19	1.792E-16
Generation of neurons	1871	7.046E-19	2.474E-16
Neuron differentiation	1405	2.870E-18	9.237E-16

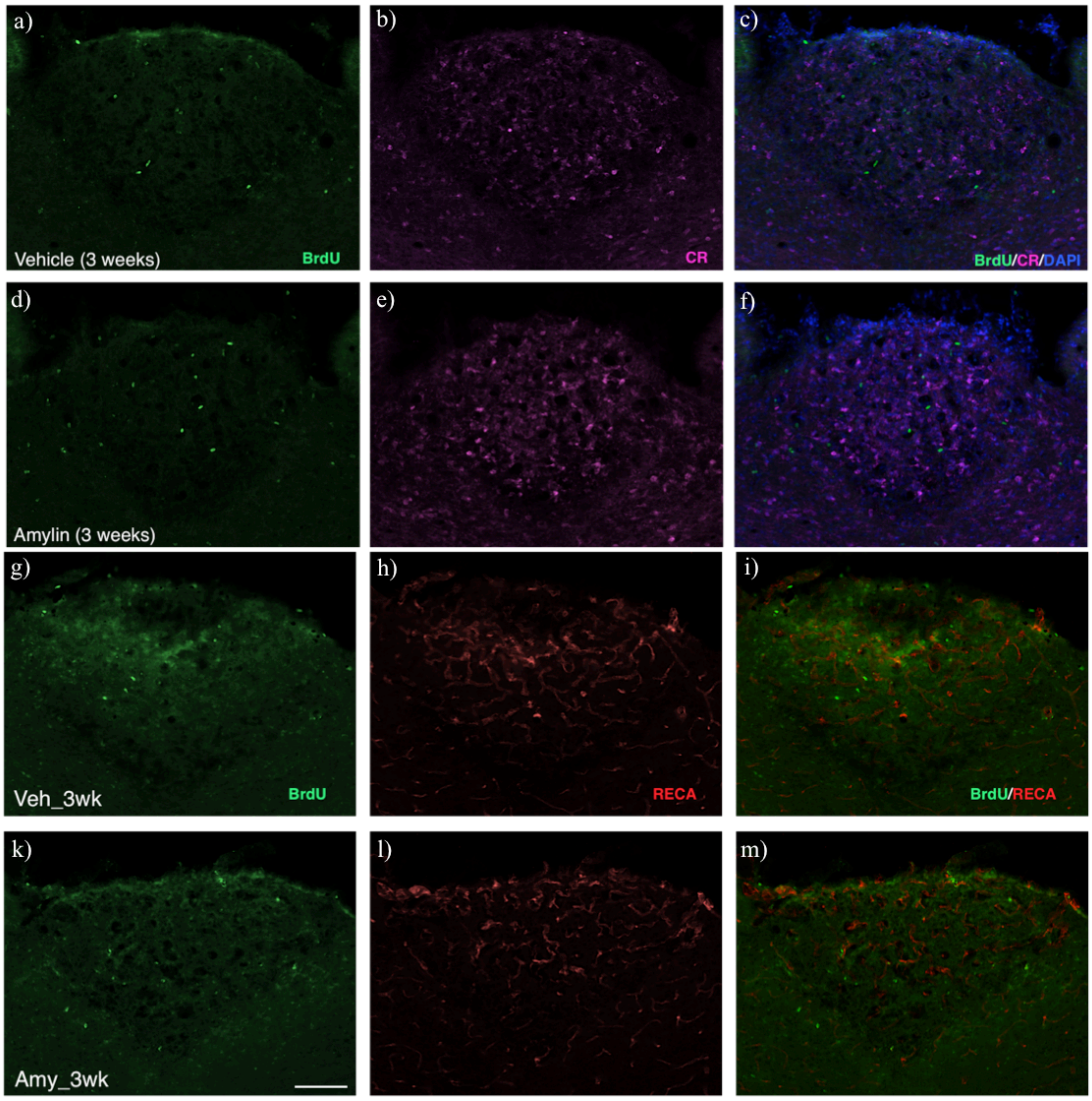
*Total refers to the total number of genes involved in a process. FRD and p-value refer to processes involved in the experimental conditions. A p-value < 0.01 is considered significant.

Supplementary Figures



Supplementary Figure 1

Amylin's effect on food intake and body weight in animal sacrificed after one or three weeks. Average daily cumulative food intake over the 1-week (a) and 3-weeks (c) treatment period was significantly decreased by amylin (Student t-test; $^{*}P < 0.05$). No difference in body weight was observed throughout the course of the experiment was observed (b, d). Data is expressed as mean ± S.E.M.



Supplementary Figure 2

BrdU-labeled cells in the AP of vehicle (a,g) and amylin (d,j) treated rats (*n* = 8 *per* group); amylin was given for 3 weeks at a dose of 50 µg/kg/day. Sections stained for interneurons (CR) and blood-vessels (RECA-1) markers, respectively, are shown in (b,e,h,l). BrdU-positive neurons double labelled for CR (c, f) and RECA-1 are shown in (c,f) and (i,m), respectively. No co-localization was observed. Scale bar represents 50 µm.

7. Unpublished results

In this section I will briefly summarize preliminary data collected with the double transgenic *Nestin/hRAMP1* mice. The aim of this pilot study was to provide a basic characterization of the feeding behavior of the double transgenic mice maintained on regular chow and on HFD. Further, to investigate the potential role of RAMP1 to enhance amylin and/or leptin central signaling, we performed crossover studies on two cohorts of animals and evaluated the meal pattern after each treatment. The brains of the *Nestin/hRAMP1* and control mice (*i.e.,* single-transgenic mice containing only the hRAMP1 or *Nestin* transgene) have been accurately preserved for future immunohistochemical analysis. Similarly, the carcasses of the animals have been stored for further CT-scan. The expected outcomes and the potential implications for our findings are discussed in ¶ 7.6.1.

7.1 Characterization of the feeding behaviour of the *Nestin/hRAMP1* double transgenic mice maintained on chow and high-fat diet.

7.2 Background

hRAMP1 is a 148 aa protein with a large extracellular domain, a single predicted transmembrane spanning domain and a short cytoplasmic domain (Mallee et al., 2002). The co-expression of RAMP1 with CTRa in the same single neuron, *e.g.* in the rat AP (Liberini et al., 2016), generates the AMY_1, whereas the coupling of RAMP1 with CLR generates CGRP receptors (McLatchie et al., 1998). The double transgenic mouse model *Nestin-hRAMP1* expresses the human RAMP1 transgene in the brain, trigeminal ganglion, spinal cord and dorsal root ganglion (Zhang et al., 2011). Previous findings have shown that *Nestin/hRAMP1* mice have reduced body weight, reduced adiposity and increased energy expenditure (Zhang et al., 2011). Thus, these double transgenic mice are characterized by a marked lean phenotype. It has been shown that both amylin and CGRP peptides reduce food intake and body weight (Lutz, 2006; Lutz et al., 1998a; Morley et al., 1996). Indeed, after *intracerebroventricular* (ICV) administration, amylin and CGRP decreased body weight and food intake in control animals, while *Nestin/hRAMP1* mice showed an

enhanced response to amylin but not to CGRP. However, CGRP mRNA was elevated in the hypothalamus of the double transgenic mice (Zhang et al., 2011). These findings suggest that RAMP1 might enhance the central signaling of peptides involved in the regulation of feeding behaviour, such as amylin and CGRP. Moreover, RAMP1 might protect against obesity. Therefore, *Nestin/hRAMP1* mice are an important tool to elucidate brain signaling in the context of the pathophysiology and intervention on obesity.

Finally, the ability of RAMPs to modulate numerous aspects of G-protein-coupled receptor (GPCR) signaling makes them interesting pharmacological targets that may be useful for the development of specific drugs in humans and animals. GPCRs are located at the cell surface, which makes them easily accessible therapeutic ("drugable") targets. Furthermore, they play a fundamental role in many cell types as the *sensors* or *transmitter* of extracellular chemical signals into messages resulting in cellular change. Indeed, GPCRs are, to date, among the most common types of pharmacologically-targeted molecules (Nickols and Conn, 2014; Roux and Cottrell, 2014).

7.2.1 Generation of the double transgenic *Nestin/hRAMP1* mice

Nestin/hRAMP1 mice are a double transgenic mouse model that results from the breeding of the transgenic mice *Nestin/cre* with the transgenic mice *GFP/hRAMP1*. Briefly, the *cre* gene is under the control of the nervous system specific promoter and enhancer rat *nestin* gene. The *cre*-mediated recombination system is a powerful and broadly utilized genetic approach to conditionally express (or delete) selected genes. Genotyping by PCR is required to identify the presence of the *nestin* gene and thus characterize a mouse as transgenic. *GFP/hRAMP1* mice were previously generated by using a vector which contains the ubiquitous active Chicken-X1 (CX1) promoter upstream, and a downstream GFP-stop-hRAMP1 cassette flanked by loxP sites (Zhang et al., 2007b). The presence of GFP allows a rapid genotyping. By using Dark Reader device (Seville, 2001), *GFP/hRAMP1* mice can easily be identified thanks to the green fluorescence visible on the nose, inner ears and paws. Once the mice have been correctly genotyped, male *Nestin/cre* are bred with female *GFP/hRAMP1* mice

to express the human RAMP1 transgene. The GFP cDNA has a translational stop sequence and a polyA signal that prevents expression of hRAMP1 in these mice. Because of that, hRAMP1 expression is dependent on *cre*-recombinase excision of GFP at flanking loxP sites (Figure 7). The resultant hRAMP1 transgene is then expressed from CX1 promoter only in the nervous system.

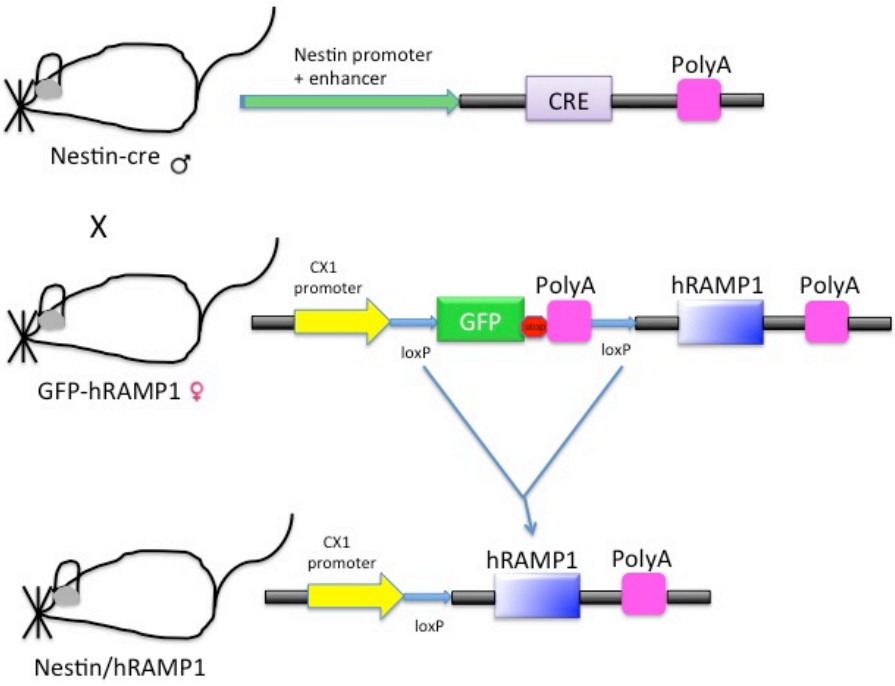


Figure 7: Breeding strategy for the generation of double transgenic *Nestin/hRAMP1* mice.

Nestin/cre male mice are characterized by the presence of the rat *Nestin* promoter and enhancer driving *cre* recombination. Male *Nestin/cre* mice are mated with female *GFP/hRAMP1* mice to obtain *Nestin/hRAMP1* progeny. *GFP/hRAMP1* has a CX1 promoter and a GFP-stop-hRAMP1 cassette flanked by loxP sites. CX1 drives ubiquitous expression of GFP, but not hRAMP1, because the GFP cDNA has a translation stop codon and a polyadenylation signal (polyA). *Cre* mediates the excision of the GFP–stop sequence at the flanking loxP sites and the resultant progeny (*Nestin/hRAMP1*) have the hRAMP1 transgene expressed from the CX1 promoter only in the nervous system. Adapted from (Zhang et al., 2007a).

7.3 Aim

To further characterize the feeding behaviour of *Nestin/hRAMP1* transgenic mice, we performed meal pattern analysis using the BioDaq system which measures food intake, number of meals and meals size. Additionally, to evaluate a potential role of central RAMP1 in the interaction of amylin and leptin signaling (Roth et al., 2008; Trevaskis et al., 2010a), crossover studies were performed. Finally, to investigate whether an increased RAMP1 signaling might protect against obesity, we challenged the mice with high-fat diet (HFD; 60% fat with 0.25% added cholesterol; Research Diet, New Brunswick, NJ, USA). Meal pattern analysis was performed and energy expenditure was measured using an indirect calorimetry system.

7.4 Design

Experiment 1: *Nestin/hRAMP1* mice (8-12 weeks old, *n*=7-8 *per* group) were acclimated for a week to the BioDaq Monitoring System before any data was collected. Animals had *ad libitum* access to water and standard chow (no. 3430; Provimi Kilba, Gossau, Switzerland) unless otherwise stated. To acquire baseline measurements on chow, data were recorded for six days and the animals were manually weighted daily. Crossover experiments (vehicle *versus* amylin [50 µg/kg, *i.p*], vehicle *versus* leptin [5 mg/kg, *i.p*] and vehicle *versus* amylin plus leptin, *i.p*) were carried on to the same cohort of animals with a three-day interval between trials. Prior to treatments, animals were fasted for two hours in the light phase and injected at dark onset. Feeding behavior parameters, such as food intake, number of meals and meal size were analyzed at 1, 2, 4, 12 and 24 hour post injections. Here, data are shown at 4 and 24 hours time points (Figure 8a).

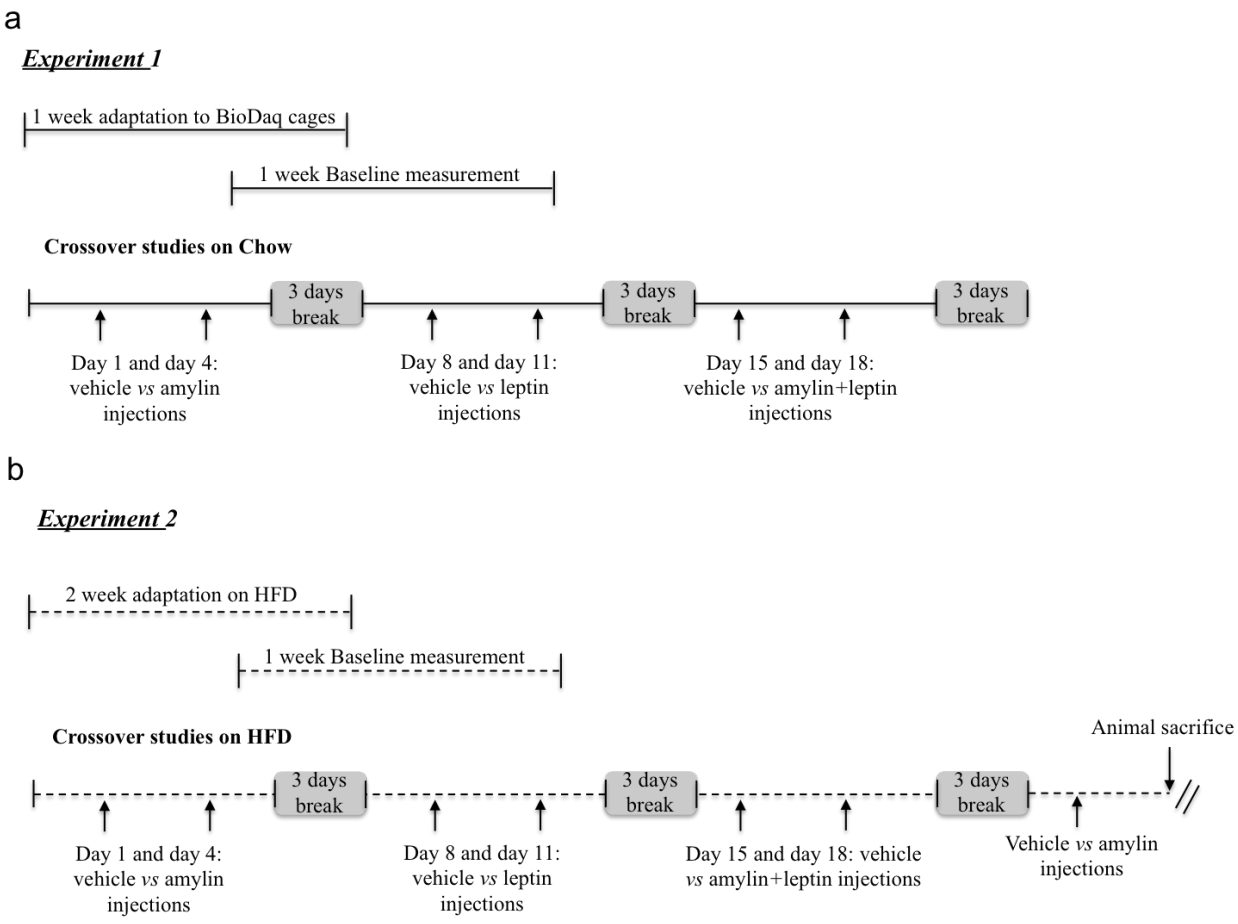


Figure 8: Experimental plan

Experiment 1: double transgenic *Nestin/hRAMP1* mice and control (*n*= 6-7 *per* group) from the same cohort were initially adapted for a week to the BioDaq cage system. Mice were maintained on normal chow. After a week of baseline measurement crossover experiments with amylin (50 µg/kg), leptin (5 mg/kg) and amylin plus leptin were performed. In *Experiment 2*, animals were adapted for two weeks to HFD. After a week of baseline measurement crossover experiments with amylin, leptin and amylin plus leptin were performed. Animals were finally treated with vehicle or amylin and sacrificed.

Experiment 2: the same cohort of animals from *Experiment 1* was then adapted for two weeks to HFD. To acquire baseline measurements on HFD, data were recorded for a week and animals were manually weighted daily. Crossover studies (vehicle *versus* amylin [50 µg/kg, *i.p*], vehicle *versus* leptin [5 mg/kg, *i.p*] and vehicle *versus* amylin and leptin, *i.p*) were performed as described in *Experiment 1*. At the end of the experiment, mice were acutely treated with vehicle or amylin (50 µg/kg, *i.p*)

(Figure 8b). Forty-five minutes after injections animals were deeply anesthetized with an *i.p*. injection of sodium pentobarbital (100 mg/kg) and transcardially perfused using 0.1M PBS followed by 4% paraformaldehyde (PFA).The brains were collected and maintained overnight in 4% PFA. Subsequently, the brains were transferred to 20% sucrose in PBS for 24 hours. Brains were cut into 20-µm coronal sections on a cryostat (Leica microsystem, Wetzlar, Germany) and stored in cryoprotectant (20% glycerol, 30% ethylene glycol, 50% 0.1M PB) until processing for immunohistochemistry. The carcasses of the animals were stored at -20°C for further CT-scan analysis. Data were analysed by two-way ANOVA followed by Bonferroni post-hoc test to find differences between groups. Data are represented as mean ± S.E.M and statistical significance was established at $P < 0.05$.

Experiment 3: for the metabolic study, *Nestin/hRAMP1* mice (9-14 weeks old, *n*=6 *per* group) were single housed in metabolic cages (TSE Phenomaster, TSE Systems, Germany) and adapted to the housing conditions for six days before starting the experiment. Animals had *ad libitum* accesses to water and HFD unless otherwise stated. Body weight and food intake were manually measured daily. A crossover study was performed: vehicle *versus* amylin (50 µg/kg, *i.p*) was administered to the mice at two-day intervals. Three days later, the same cohort of animals was treated with leptin (5 mg/kg, *i.p*) and sacrificed as described in *Experiment 2*. Brains and carcasses of the mice were stored at -20°C for further immunohistchemical and CT-scan analysis. The respiratory exchange ratio (RER) and energy expenditure (EE) were calculated from O_2 consumption and CO_2 production as described previously (Riediger et al., 2010). EE data were normalized for body weight. The calculation was based on the following equation: total EE (kcal/kg/h) = $(3.9 \times V_{O2} + 1.1 \times V_{CO2}) / 1000$; RER = V_{CO2} / V_{O2}. The day just before the injections was used as baseline and compared to the values obtained during the treatment period. Group means were analyzed with Student t-test. Data are represented as mean ± S.E.M and $P < 0.05$ was considered significant.

7.5 Results

7.5.1 Nestin/hRAMP1 mice maintained on chow have reduced body weight, decreased food intake and a reduced number of meals per day.

To investigate the role of the expression of neuronal hRAMP1, body weight, food intake, the number of meals per day and the meal size were monitored daily *via* the BioDaq system in *Nestin/hRAMP1* double transgenic and control mice. *Nestin/hRAMP1* mice showed a marked reduction in body weight ($^{*}P < 0.05$) over time and decreased food intake (Student t-test; $t_{13}=2.350$; $^{*}P = 0.0352$) compared to controls (Figure 9 a,b). Detailed analysis of feeding behavior revealed that the number of meals per day was significantly lower in *Nestin/hRAMP1* mice (Student t-test; $t_{13}=2.582$; $^{*}P = 0.0228$; Figure 9c) compared to control, whereas the meal size (grams ate/meal/mouse) was increased (Student t-test; $t_{13}=2.276$; $^{*}P = 0.0404$; Figure 9d).

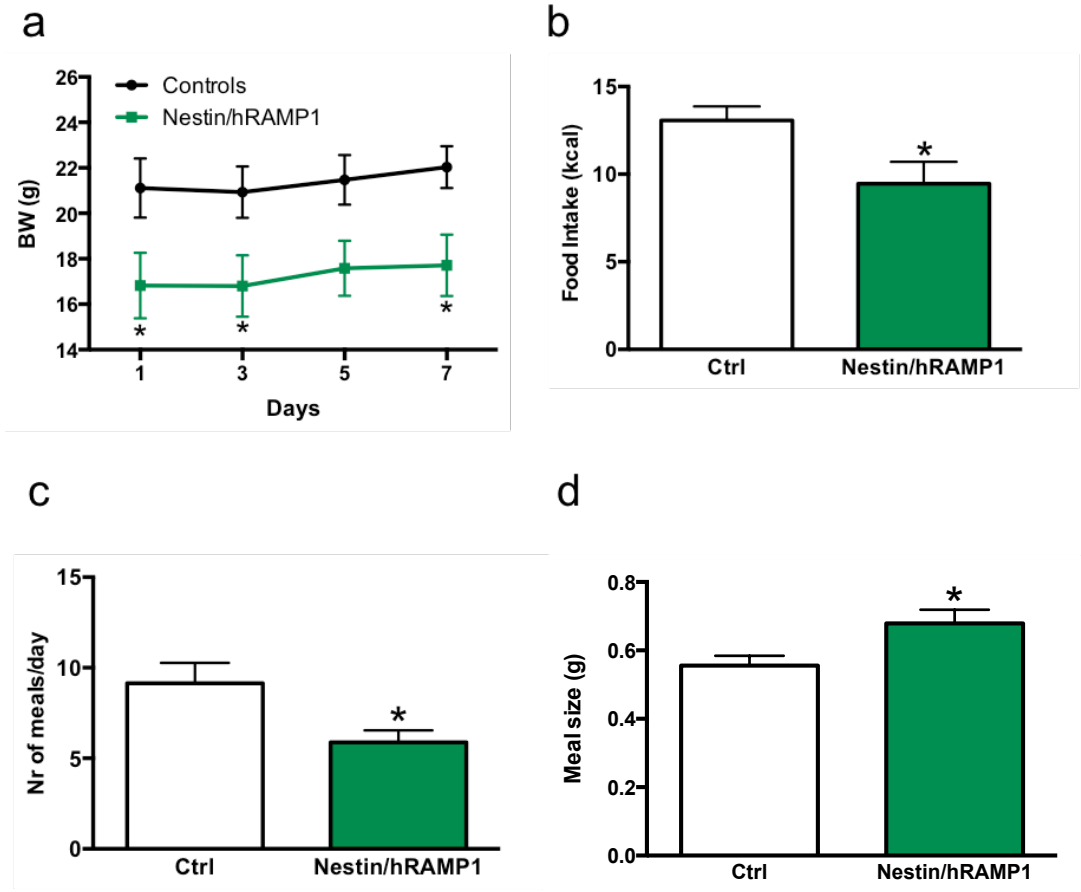


Figure 9: Feeding behavior of *Nestin/hRAMP1* mice maintained on chow.

Baseline measurements of body weight, food intake and meal pattern analysis of *Nestin/hRAMP1* mice maintained on chow. (a) Body weight was significantly lower in double transgenic mice, compared to controls (Student t-test; $^{*}P<0.05$). (b) *Nestin/hRAMP1* mice ate significantly less (Student t-test; $^{*}P<0.05$), (c) ate fewer meals per day (Student t-test; $^{*}P<0.05$) but (d) had increased meal size (Student t-test; $^{*}P<0.05$), compared to controls. Data is represents as mean ± S.E.M.

7.5.2 Genotype significantly influenced the feeding behavior of Nestin/hRAMP1 mice maintained on chow. Moreover, the co-administration of amylin plus leptin significantly decreased food intake in the double transgenic mice.

The genotype *per se* influenced the general feeding behavior of the double transgenic mice after all the individual treatments (Two-way ANOVA; $P<0.05$, Figures 10,11,12). Acute amylin administration did not affect body weight, food intake,

number of meals consumed, or meal size, in *Nestin/hRAMP1* mice or control; at any time point over the next 24 hours (here, shown at 4 and 24 hours; Figure 10). Unexpectedly, amylin treatment failed to reduce food intake.

Leptin treatment and genotype significantly decreased food intake at 24h but not at 4h (Two-way ANOVA; $P<0.05$, Figure 11b) in double transgenic mice and a trend is present for the slightly diminished body weight (Figure 11a) and reduced number of meals (Figure 11c). Amylin and leptin co-administration significantly reduced food intake in *Nestin/hRAMP1* mice at 24h (Bonferroni's Post-hoc test; $^{*}P<0.05$; Figure 12b). The genotype also affected the decreased in body weight and in food intake at 4h and 24h (Two-way ANOVA; $^{*}P<0.05$, Figure 12a,b) in *Nestin/hRAMP1* mice but no effect on number of meals (Figure 12c) nor on meals size (Figure 12d) was observed.

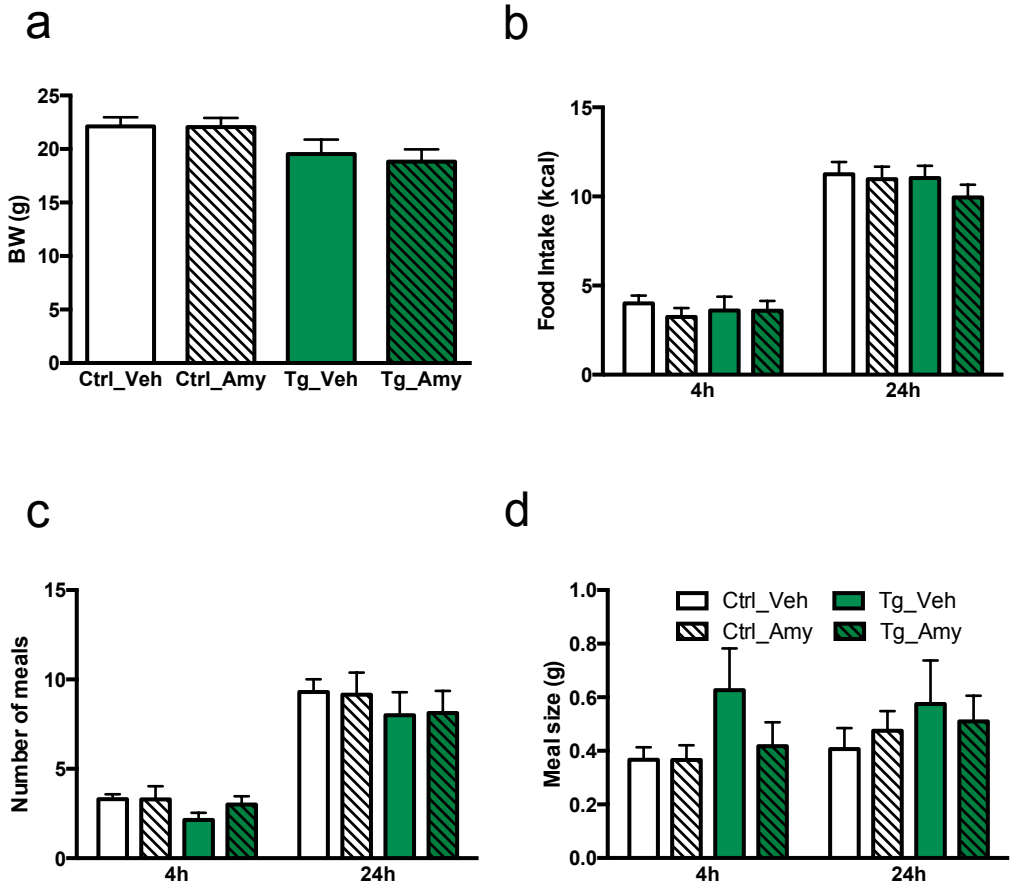


Figure 10: Meal pattern analysis after amylin treatment in mice maintained on chow.

Body weight, food intake and meal pattern analysis of *Nestin/hRAMP1* mice maintained on chow after acute amylin treatment (50 µg/kg). The genotype *per se* reduced (a) body weight, (b) food intake, (c) number of meals and (d) meal size (Two-way ANOVA; $P<0.05$) in *Nestin/hRAMP1* mice, while no effect of treatment or interaction between genotype and treatment was observed. Data is represents as mean ± S.E.M.

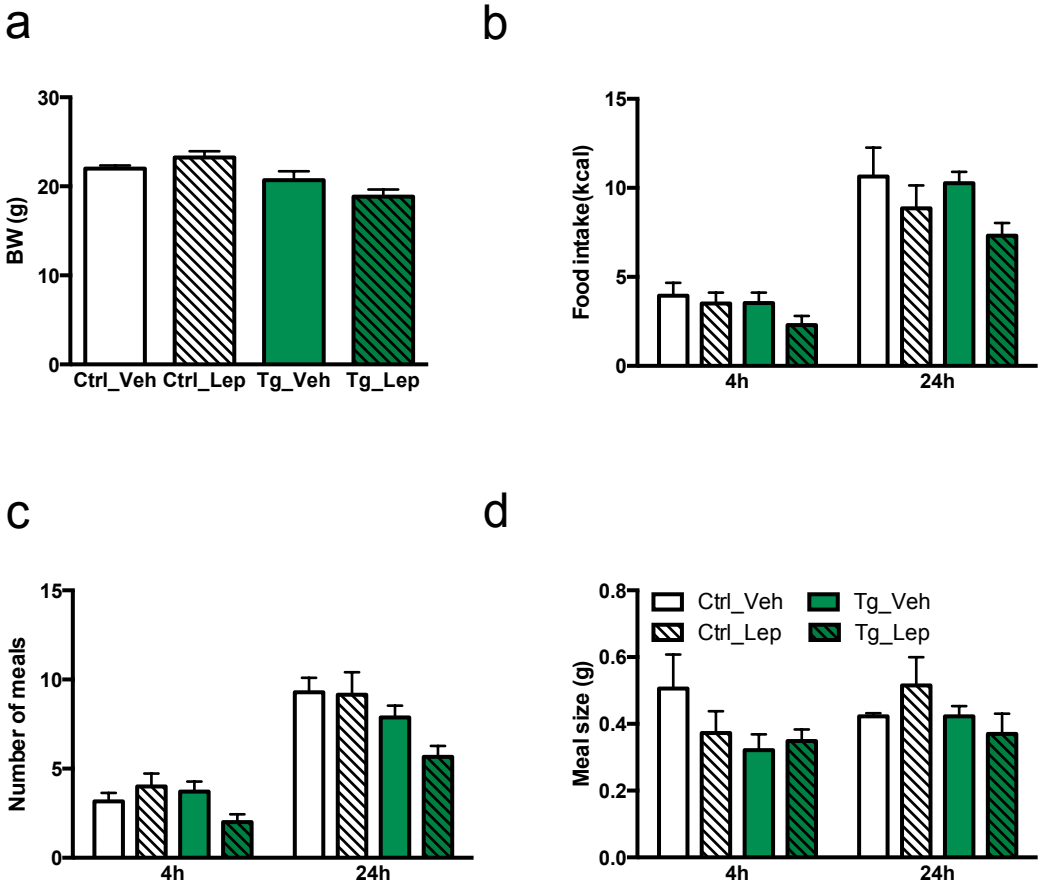


Figure 11: Meal pattern analysis after leptin treatment in mice maintained on chow.

Body weight, food intake and meal pattern analysis of *Nestin/hRAMP1* mice maintained on chow after acute leptin treatment (5 mg/kg). (a) The body weight was lower in the double transgenic mice (Two-way ANOVA; $P<0.05$). (b) Genotype and treatment decreased the food intake (Two-way ANOVA; $P<0.05$) and reduced (c) the number of meals at 24h (Two-way ANOVA; $^{*}P<0.05$) in *Nestin/hRAMP1* mice, while no significant difference was observed (d) in the meal size. Data is represents as mean ± S.E.M.

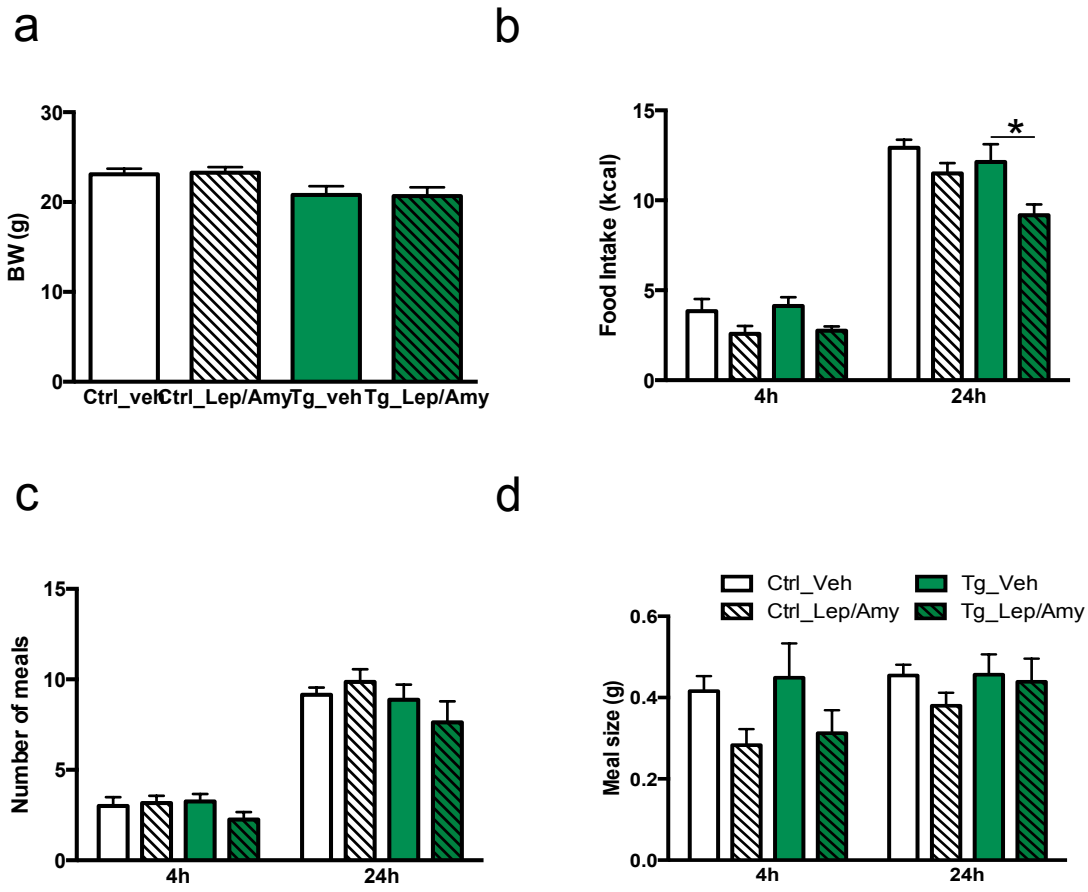


Figure 12: Meal pattern analysis after amylin plus leptin treatment in mice maintained on chow.

Body weight, food intake and meal pattern analysis of *Nestin/hRAMP1* mice maintained on chow after acute amylin (50 µg/kg) and leptin treatment (5 mg/kg). (a) A significant effect of genotype was observed on body weight (Two-way ANOVA; $P<0.01$) in *Nestin/hRAMP1* mice. (b) Genotype significantly affected food intake at 4h and 24h (Two-way ANOVA; $P<0.05$); at 24h, the administration of amylin plus leptin reduced the food intake (Two-way ANOVA; $P<0.01$) and food intake was significantly reduced by amylin and leptin in double transgenic mice (Bonferroni's; $^{*}P<0.05$). Neither amylin plus leptin treatment nor genotype affected the (c) number of meals or (d) meal size. Data is represents as mean ± S.E.M.

7.5.3 Nestin/hRAMP1 mice maintained the lean phenotype after HFD challenge. Moreover, hRAMP1 seemed to slightly enhance leptin signaling.

To investigate whether central hRAMP1 can protect against obesity, animals were challenged with HFD. Feeding behavior analysis showed that *Nestin/hRAMP1* mice maintained a reduced body weight through the entire experiment (Student t-test; $^{*}P <$

0.05; Figure 13a). No significant difference was observed in food intake, number of meals per day or meal size (Figure 13 b,c,d).

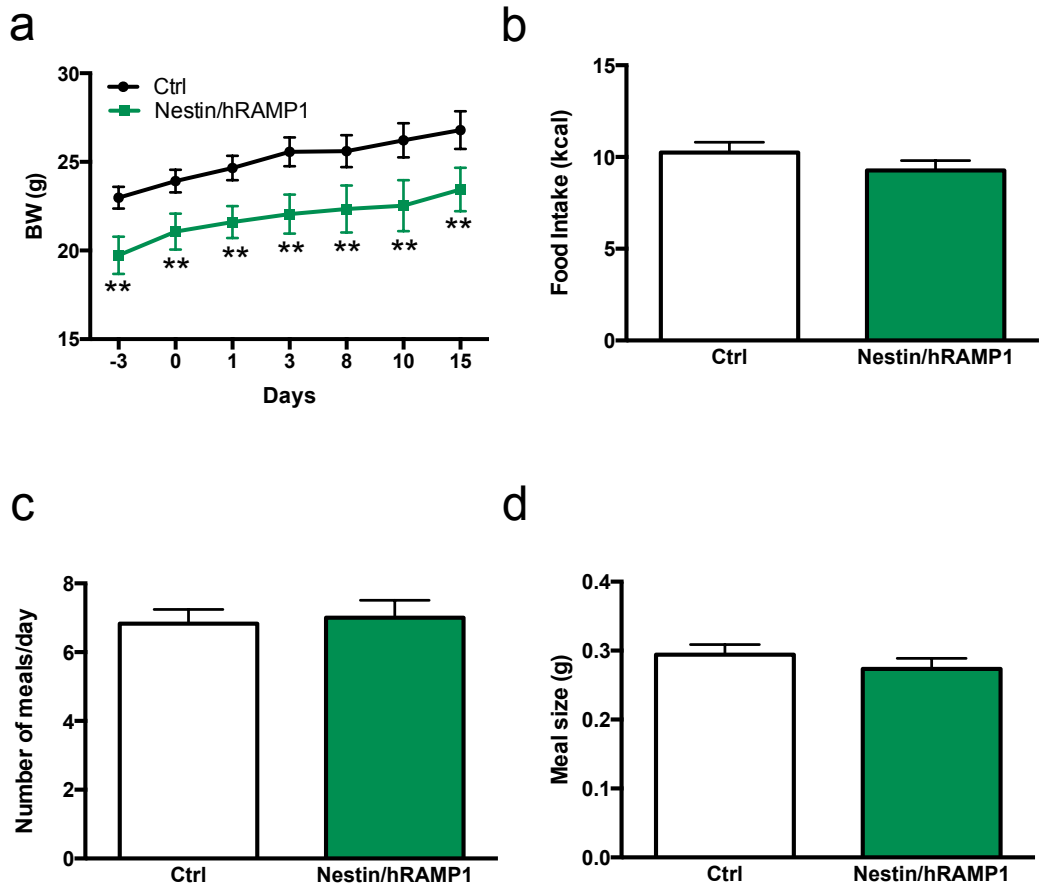


Figure 13*:* Feeding behavior of *Nestin/hRAMP1* mice maintained on HFD.

Body weight, food intake and meal pattern analysis of *Nestin/hRAMP1* mice maintained on HFD. (a) Body weight was significantly lower in double transgenic mice, compared to control (Student t-test; $^{*}P<0.05$). No difference in (b) food intake, (c) number of meals per day nor (d) meal size was observed. Data is represents as mean ± S.E.M.

Amylin treatment had no effect on the feeding behavior in the double transgenic mice or control while the genotype *per se* affected the decrease in body weight and in food intake at 4h and 24h (Two-way ANOVA; $P<0.05$; Figure 14a,b). No significant difference was observed in number of meals, nor meal size (Figure 14c,d). Genotype

per se reduced body weight after leptin administration (Two-way ANOVA; $P<0.05$, Figure 15a) in *Nestin/hRAMP1* mice. Leptin treatment significantly reduced food intake at 24h, but not at 4h (Two-way ANOVA; $P<0.05$, Figure 15b) while no effect of the genotype was observed at neither time point. Neither the number of meals nor the meal size were affected by the treatment or the genotype at 4h and 24h (Figure 15c,d). Finally, animals on HFD received an injection of amylin plus leptin. In *Nestin/hRAMP1* mice the genotype significantly reduced the body weight (Two-way ANOVA; $P<0.01$, Figure 16a). The combined treatment of amylin plus leptin decreased food intake (Two-way ANOVA; $P<0.01$, Figure 16b) at 4 and 24h in double transgenic mice. Moreover, in control animals leptin significantly reduced food intake (Bonferroni's post-hoc test; $^{*}P<0.01$, Figure 16b) at 24h. In *Nestin/hRAMP1* mice, amylin plus leptin treatment also reduced the number of meals at 4h and 24h (Two-way ANOVA; $P<0.01$, Figure 16c). Neither the genotype nor the treatment affected the meal size (Figure 16d).

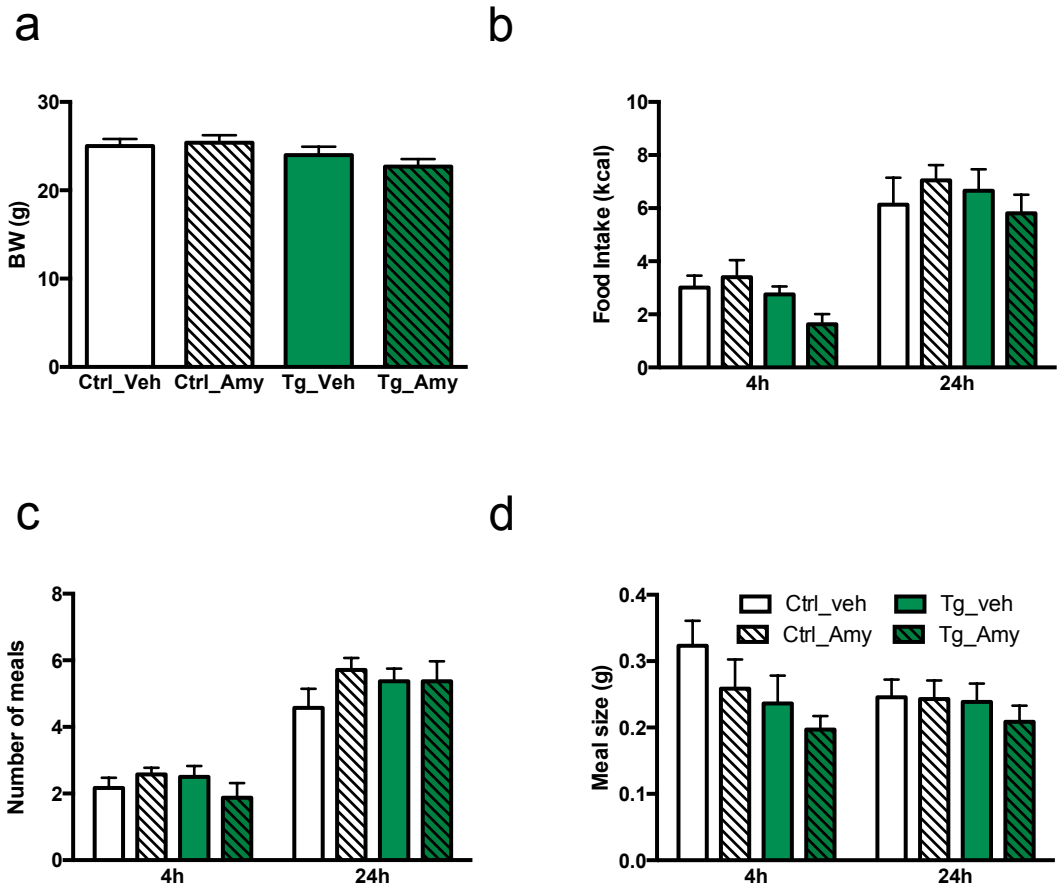


Figure 14: Meal pattern analysis after amylin treatment in mice maintained on HFD.

Body weight, food intake and meal pattern analysis of *Nestin/hRAMP1* mice maintained on HFD, after acute amylin treatment (50 μg/kg). (a) Body weight and (b) food intake at 4h but not 24h, was decreased in double transgenic mice compared to control (Two-way ANOVA; $P<0.05$). No effect of genotype or treatment was observed in (c) the number of meals or (d) in the meal size. Data is represents as mean ± S.E.M.

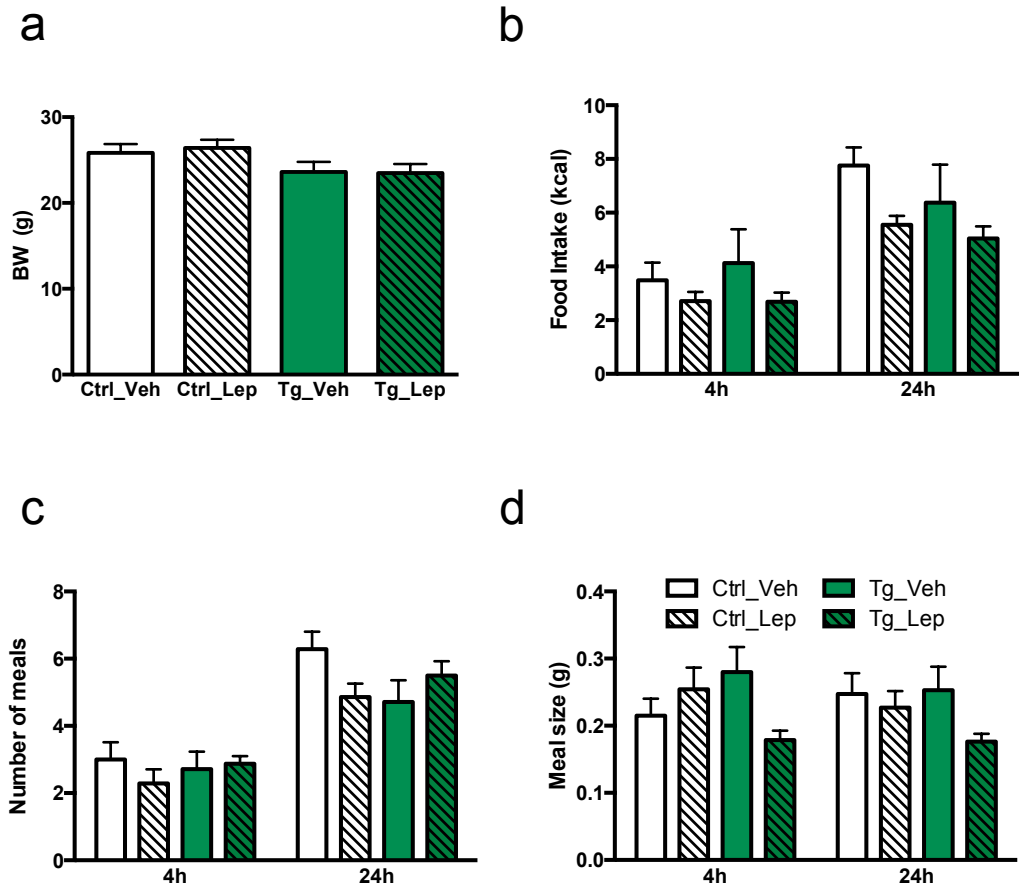


Figure 15: Meal pattern analysis after leptin treatment in mice maintained on HFD.

Body weight, food intake and meal pattern analysis of *Nestin/hRAMP1* mice maintained on HFD, after acute leptin treatment (5 mg/kg). (a) Body weight was decreased by the genotype *per se* (Two-way ANOVA; $P<0.05$). (b) Leptin treatment significantly affected the reduction in food intake at 24h, but not at 4h (Two-way ANOVA; $P<0.05$) while no effect of the genotype was observed at either time point in *Nestin/hRAMP1* mice. (c) Neither the number of meal nor (d) meal size were affected by treatment or genotype at 4h and 24h. Data is represents as mean ± S.E.M.

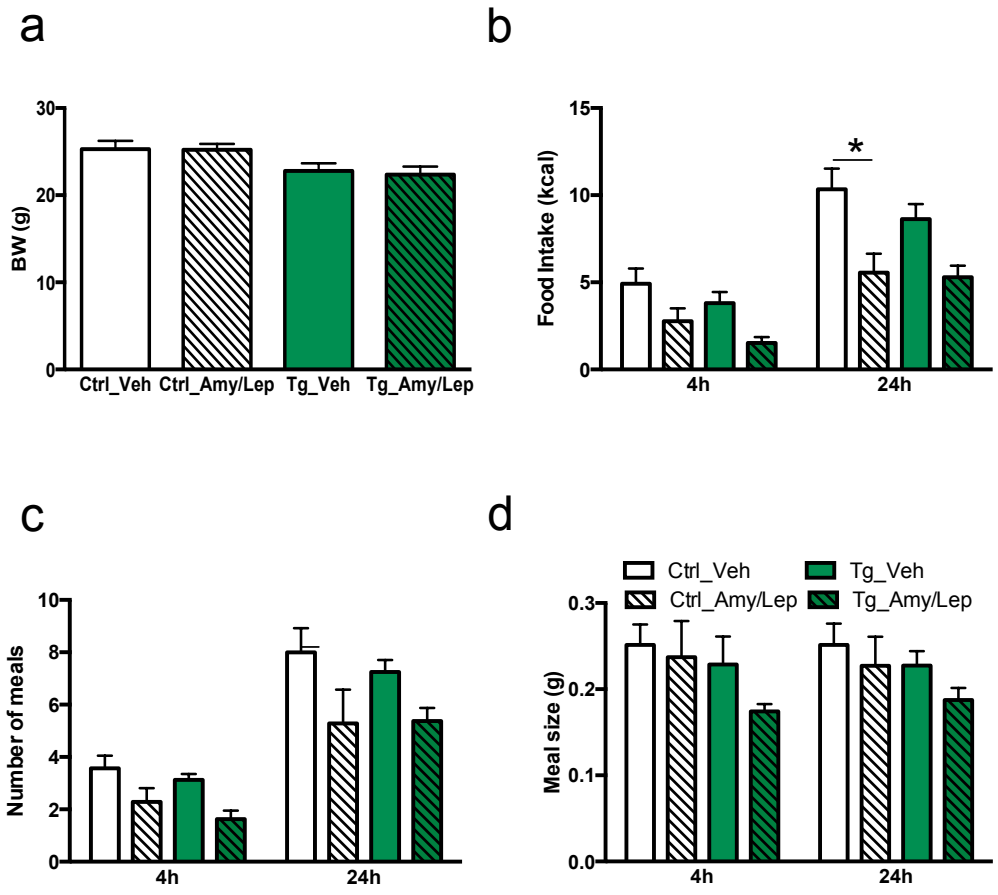


Figure 16: Meal pattern analysis after amylin plus leptin treatment in mice maintained on HFD.

Body weight, food intake and meal pattern analysis of *Nestin/hRAMP1* mice maintained on HFD, after amylin (50 µg/kg) and leptin (5mg/kg) treatment. (a) The genotype *per se* significantly reduced the body weight (Two-way ANOVA; $P<0.01$). (b) The combined treatment of amylin plus leptin decreased the food intake (Two-way ANOVA; $P<0.01$) at 4h and 24h. In control animals, leptin significantly reduced food intake (Bonferroni's post-hoc test; $^{*}P<0.01$) at 24h. (c) Amylin plus leptin treatment reduced the number of meal at 4h and 24h (Two-way ANOVA; $P<0.01$). (d) Neither the genotype nor the treatment affected the meal size. Data is represents as mean ± S.E.M.

7.5.4 Nestin/hRAMP1 mice maintained on HFD seemed to have increased energy expenditure.

To assess the potential effect of the neuronal expression of hRAMP1 on energy expenditure, mice were housed in metabolic cages. Double transgenic mice showed lower EE over 24h compared to control (Figure 17a). Furthermore, *Nestin/hRAMP1* mice seemed to maintain lower EE in both light and dark phases (Figure 17c).

However, when data was corrected for the body weight, our results demonstrated that the double transgenic mice actually displayed a significant increase in EE compared to control (Figure 17b), and this difference was maintained in both light and dark phases (Figure 17d).

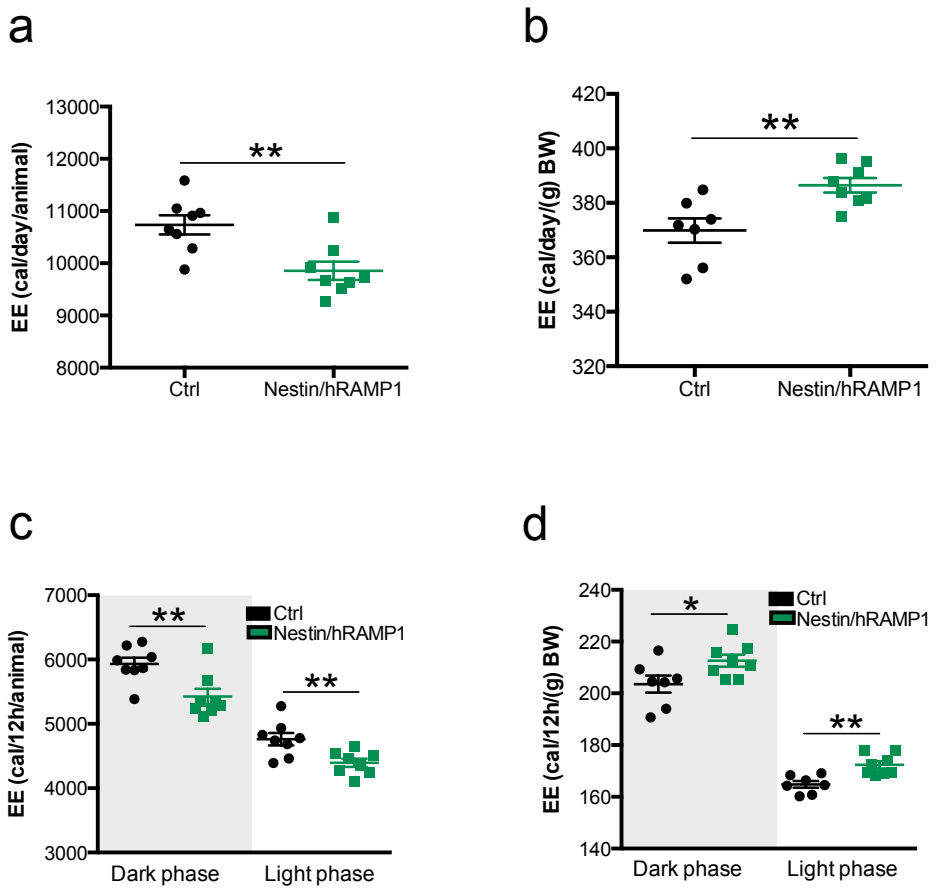


Figure 17: Energy expenditure analysis of mice maintained on HFD.

(a) Daily EE (cal/day/animal) of the *Nestin/hRAMP1* mice was significantly lower compared to controls. (b) EE corrected for the body weight was significantly higher in double transgenic mice compared to control. (c) In *Nestin/hRAMP1* mice EE was lower during both light and dark phases (12h/12h). (d) EE corrected for body weight was significantly higher in transgenic mice compared to controls. Data was analyzed using the Student's t test; * $P < 0.05$, ** $P < 0.01$. Data is represents as mean ± S.E.M.

7.6 Conclusions and future perspectives

Our current work confirmed that *Nestin/hRAMP1* mice have a marked lean phenotype (Zhang et al., 2011). Moreover, the meal pattern analysis showed that *Nestin/hRAMP1* mice have decreased food intake, reduced number of meals per day and increased meal size; thus supporting the role of RAMP1 in the regulation of energy metabolism. While ICV injection of amylin reduced food intake to weight gain ratio in previous studies (Fernandes-Santos et al., 2013; Zhang et al., 2011), unexpectedly, here, peripheral amylin administration failed to decrease feeding. *Nestin/hRAMP1* mice express the hRAMP1 in addition to the constitutive mouse RAMP1, and this results in an overexpression of RAMP1 in the CNS. Recently, we demonstrated that acute amylin treatment down-regulated RAMP1 mRNA in single AP-neurons, suggesting that amylin self-modulates the accessory subunits of its own receptor in this brain area (Liberini et al., 2016). Whether a similar mechanism happens in other brain nuclei is not known yet. We speculate that one possibility for the lack of amylin effect might be that the overexpression of RAMP1 may lead to a diminished response in the AP-neurons. Thus resulting in a blunted amylin effect to reduce food intake. Further studies are still required to investigate the long-term behavior of the RAMPs in response to amylin.

However, the genotype *per se* significantly affected the general feeding behavior of *Nestin/hRAMP1* mice. Similarly, leptin treatment displayed a trend in the reduction of food intake, body weight and number of meals consumed in *Nestin/hRAMP1* mice, although it did not reach statistical significance. The co-administration of amylin plus leptin reduced food intake in double transgenic mice, suggesting a potentiation of the synergistic effect previously described in non-transgenic rodents (Roth et al., 2008; Trevaskis et al., 2010b; Turek et al., 2010). It remains to be addressed which brain areas are involved in the maintenance of the lean phenotype and where the amylin-leptin interaction takes place. A potential candidate appears to be the AP. Indeed, recent work from our group demonstrated that 30% of single amylin-activated AP-neurons co-expressed the LepRb mRNA, in addition to the mRNA of all the necessary components to form a functional AMY (Liberini et al., 2016). This suggests the existence of a first-order neuronal population in the AP that is responsive to both

amylin and leptin. Further, LepRb expression was up-regulated after acute amylin treatment in single AP-neurons (Liberini et al., 2016), indicating that amylin might enhances leptin sensitivity in this brain area. However, the VMH and the VTA could also play a role in amylin-leptin interaction (Le Foll et al., 2015; Li et al., 2015; Mietlicki-Baase et al., 2015a). Fos immunohistochemistry studies or studies using other activation markers to identify the central pathways activated by amylin and/or leptin are still missing.
A limitation of this mouse model is that the expression of the hRAMP1 is not restricted to any specific brain nuclei but it is expressed throughout the entire CNS. Therefore, neurons that ectopically carry RAMP1 in different brain areas, might also contribute to the enhanced metabolic effect. A viral-mediated approach would be the strategy to selectively study the effect of RAMP1 in specific brain areas.

Finally, we also demonstrated that the characteristic lean phenotype of the *Nestin/hRAMP1* is maintained when animals are challenged on HFD and this is possibly the result of an increase in EE. However, our data are only preliminary and describe the situation in the short-term period. Long-term studies would be instrumental to further characterize the role of RAMP1 to possibly protect from obesity. Moreover, bodies CT-scan analysis is required to evaluate the distribution of the lean and the fat mass and to correct the metabolic rate for lean mass. Furthermore, excluding a role of locomotor activity and core temperature would be essential to fully characterize the maintenance of the lean phenotype.

All together, these data suggest that, despite the diet, *Nestin/hRAMP1* mice are leaner compared to their control and that the reduced body weight is the result of an increased EE rather than a decrease in food intake. Hence, the *Nestin/hRAMP1* mice seem to be an effective model to investigate RAMP1-mediated effect on energy homeostasis.

7.6.1 Outlooks

As mentioned in the experimental design section, brains and carcasses of the *Nestin/hRAMP1* mice have been store for future investigation.

In this section I will describe the expected outcomes.

Outlook 1

c-Fos is a well known marker of neuronal activation. Amylin induced-Fos response has been documented in several brain areas, including the AP (Braegger et al., 2014; Potes et al., 2010b). *Experiment 2* was aimed to determine amylin-induced patterns of *c-Fos* activation in the brains of double transgenic mice expressing hRAMP1 and in non-transgenic controls, maintained on HFD**.** Since the AP is a principle site of amylin action, and together with the hypothalamus seems also to be an area where amylin and leptin interaction might occur (Le Foll et al., 2015; Li et al., 2015; Mietlicki-Baase et al., 2015a), Fos-IHC will be investigate in these brain nuclei. We hypothesized that amylin administration would result in an increased neuronal activation in the AP and in some hypothalamic areas (*e.g;* VMH and LH) in *Nestin/hRAMP1* mice. Further, to confirm that the Fos-activated neurons potentially bear an AMY, we aim to perform a double staining of the tissue sections for Fos/CTR. CTR is the core subunit of the AMY and would be indicative of the presence of AMYs. Unfortunately, good RAMP antibodies are not commercially available. We expect to find a broad overlap of Fos and CTR staining, demonstrating that AMYs are present and mediate the amylin effect in these brain areas.

Outlook 2

Obesity is typically associated with leptin-resistance (Shimizu et al., 2007) and the failure in this signaling system broadly contributes to weight gain. Following close examination of basal ingestive behavior in *Nestin/hRAMP1* mice maintained on HFD, leptin response would be investigated by measuring pSTAT3, a known marker for leptin-induced activation. We speculate that *Nestin/hRAMP1* mice would show an increased leptin-sensitivity. Moreover, if activation of AMYs is somehow permissive for leptin's effect, we expect that hRAMP1 expression would enhance pSTAT3 signaling in both hypothalamic and hindbrain nuclei.

Outlook 3

The maintenance of the homeostasis is fundamental in the control of energy balance. Several lines of evidence demonstrate that acute and chronic amylin administration increases EE in rodents maintained on chow (Mack et al., 2007; Roth et al., 2006). Moreover, preliminary results from *Experiment 3* demonstrated that *Nestin/hRAMP1* mice have an increased EE on HFD. The carcasses will be CT-scanned to evaluate the body composition of fat and lean mass. We expected to find a reduction in the fat mass but not in the lean mass of the double transgenic mice. Finally, the same cohort of animals was acutely treated with amylin. Our hypothesis is that amylin would increase EE in the transgenic mice.

8. Discussion

Since the individual studies have been discussed in detail in the respective sections, I will now only briefly summarize the general results and focus on potential links between the studies and try to draw general conclusions from the entire set of experiments.

Obesity represents a high risk for the health status, and in 2014 it affected up to 40% of the population (WHO, 2014). Excessive fat mass is associated with an increased risk of cardiovascular, metabolic and gastrointestinal comorbidities (Haslam and James, 2005). Moreover, severe adiposity is also known to affect the brain by increasing the risk to develop neurodegenerative disorders (Arvanitakis et al., 2004; de la Monte and Tong, 2014). Despite the influence of social and other factors, a complex physiological system is in charge of the maintenance of the energy balance. Hormones involved in the control of feeding behavior, such as insulin, amylin, cholecystokinin, leptin, ghrelin and glucagon-like peptide-1 (to mention the most important ones) inform the brains about the overall energy status of the body and all interact to maintain the equilibrium. A deep understanding of the physiological processes regulating energy balance is essential not only to fight obesity, but also to shed light on how obesity affects the brain.

The satiating hormone amylin exerts its actions mainly by binding to the AMY in the AP (Potes and Lutz, 2010; Riediger et al., 2001). Amylin decreases food intake and body weight by reducing the number of meals without any compensatory effect on the meal size (Lutz et al., 1995). Like insulin and leptin, amylin also shares the characteristics of an adiposity signal. In fact, chronic amylin treatment clearly reduced body weight gain, specifically by decreasing fat mass, in both rodents and humans (Arnelo et al., 1996; Mack et al., 2007; Roth et al., 2006). Moreover, central amylin administration increased energy expenditure *via* a mechanism that might involve the activation of the sympathetic nervous system, which results in an increased thermogenic activity of the BAT (Fernandes-Santos et al., 2013; Zhang et al., 2011).
Despite its main role in feeding behavior, amylin has recently been shown to act as a neurotrophic factor: it induces the formation of neuronal projection from the AP to the NTS in neonatal mice (Lutz, 2010b), it recovers impaired AP and hippocampal

neurogenesis in female rats after ovariectomy (Trevaskis et al., 2010c) and the amylin analogue pramlintide restores cognitive function in a mouse model of AD (Adler et al., 2014). Hence, amylin is involved in the regulation of feeding behavior and also participates in neurogenic processes.

8.1. Effect of acute amylin administration on the AP

The two main studies described here provided new insight on the metabolic and neurogenic effects of amylin in the rodent AP. To exert its effects, amylin needs to bind to the AMYs, which are predominantly present in the AP (Potes and Lutz, 2010; Riediger et al., 2001). A functional AMY is a heterodimer of the core subunit CTRa with a member of the regulatory proteins RAMPs. Previous studies demonstrate that CTRa and the RAMPs are both present in the rodent AP (Christopoulos et al., 1999; Tilakaratne et al., 2000; Watkins et al., 2014), however evidence of co-expression of these subunits in the same cell in native tissue was still missing. Therefore, we hypothesized that CTRa and the RAMPs are co-expressed in single amylin-activated AP-neurons. Our findings revealed that in the AP, CTRa mRNA is constitutively co-expressed with one or more RAMPs mRNAs (Figure 18, point 1). Furthermore, acute amylin injection seems to modulate its own receptors: while CTRa expression remains un-affected, RAMP1 and RAMP3 are down-regulated by amylin. On the contrary, RAMP2 expression is up-regulated (Figure 18, point 2). These results support the concept that CTRa is the structural subunit of the AMY, while the RAMPs are the dynamic regulatory subunits. Moreover, amylin seems to trigger a negative feedback regulatory mechanism on its own receptors, as it was shown for other hormones, such as glucocorticoids (Rosewicz et al., 1988). Our data also depict a scenario in which more than one RAMP is normally co-expressed with CTRa in amylin-activated AP-neurons. While RAMP3 and RAMP1 transcripts were found independently or in combination with CTRa, RAMP2 co-expression with CTRa seemed to be linked to the presence of either RAMP1 and/or RAMP3. This supports the knowledge that AMY_1 and AMY_3 are the major AMYs, while AMY_2 probably function as ADM receptor in the AP (Stachniak and Krukoff, 2003). It remains to be addressed whether different physiological roles of amylin are mediated by different combination of the AMY subunits. One possibility is that each RAMP might contribute to a specific physiological effect of amylin. *Nestin/hRAMP1* mice, which express the human RAMP1, are characterized by a decreased body weight throughout

lifespan, thus displaying a marked lean phenotype (Zhang et al., 2011). The reduction in body weight seemed to be achieved by an increase in the EE rather than by a reduction of eating (Zhang et al., 2011). Indeed, our unpublished data (see ¶7.1) further support this hypothesis: *Nestin/hRAMP1* mice maintained the lean phenotype when challenged with HFD, and this resulted from an increased in EE rather than from a decreased feeding. All together these findings suggest that RAMP1 might be preferentially involved in the control of EE rather than in the control of food intake.

After acute amylin administration, RAMP2 mRNA expression was elevated in single AP-neurons. An explanation could be that RAMP2 upregulation is necessary to allow the dimerization of RAMP2 with the CLR to generate an ADM receptor over an AMY_2. *In vitro* studies demonstrated that ADM directly elicits excitatory effects on rodent AP-neurons (Allen and Ferguson, 1996). Moreover, the central action of the ADM is to delay gastric empting (Martinez et al., 1997). Amylin contributes to slow the gastric empting (Young et al., 1995), but the exact mechanism is still unknown. Therefore, we speculate that amylin might indirectly exert its effect to delay gastric empting by increasing the sensitivity of the AP-neurons to ADM.

RAMP3 was the predominant transcript co-expressed with CTRa, either alone or in combination with RAMP1 and/or RAMP2 in individual amylin-activated AP-neurons. Further, *in vitro* studies demonstrated that AMY_3 is the most potent AMY (Bailey et al., 2012). Speculating that RAMP1 might be involved in the regulation of EE and RAMP2 in the slowing of the gastric empting, RAMP3 might mediate amylin's action to decrease food intake. RAMP1 and/or RAMP3 knockdown mice are now available in our laboratory (RAMP2 knockdown model is not viable). These transgenic models would be instrumental tools to investigate the role of different RAMPs in different physiological effect of amylin.

Interestingly, 30% of the neuronal population that co-expressed all the necessary components to form a functional AMY, also co-expressed the LepRb mRNA (Figure 18, point 3). Moreover, acute amylin stimulation resulted in an increase of LepRb transcript. These findings describe a neuronal population in the AP that is responsive to both amylin and leptin, suggesting that the AP might be one of the brain sites that may mediate amylin and leptin interaction. Future studies addressing the effect of acute leptin and amylin plus leptin treatments would be instrumental to assess a

potential role of leptin to directly activate individual AP-LepRb and whether the co-administration of amylin and leptin could synergistically increase the LepRb mRNA level.

AMY subunits might not be the only factors differentially regulated by acute amylin treatment. Therefore, to provide a full picture of the acute amylin effect, we analyzed the full AP transcriptome. Interestingly, our analysis revealed that amylin essentially affected genes involved in the regulation of neurogenesis-like processes. Indeed, pathway analysis showed that Ephrin and $GABA_A$ signaling, which regulate multiple stage of neurogenesis, are the two major processes regulated by amylin.
Worthy of note, reward circuits (Dopamine-2 and Mu-opiod receptor pathways) are also affected by acute amylin treatment. It has been shown that VTA-neurons that carry AMYs, control energy balance by negative modulation of dopamine signaling in the nucleus accumbens (NAc) (Mietlicki-Baase et al., 2015b). Further, AMYs located in the shell of the NAc directly antagonized the hunger-induced behavior of mu-opioid receptors (Baisley and Baldo, 2014). Work from our group (Potes et al., 2012) demonstrated that 20% of AP-neurons characterized as pERK-positive after amylin administration are noradrenergic (DBH-neurons). These findings suggest that amylin might possibly act on AP-neurons to indirectly activate the reward circuit. Future studies are still required to deepen the role of AP-AMYs signaling in reward behavior. Moreover, results from the GO enrichment analysis demonstrated that genes affecting synaptic transmission, nervous system development and generation of neurons are also regulated by acute amylin treatment. The transcription factor *NeuroD1*, which is required in the final step of neuronal differentiation during post-natal neurogenesis, appears to be involved in all the aforementioned processes. Precisely, *NeuroD1*, is consistently up-regulated by acute amylin injection, while the administration of AC187 completely reverses this effect; supporting a direct effect of amylin on gene transcription (Figure 18, point 4). Hence, these findings highlight the potential role of amylin in the regulation of neurogenesis-like processes.

8.2 Chronic effects of amylin on the AP

Until recently, adult neurogenesis was described only in two brain areas, the SGZ (Eriksson et al., 1998; Kempermann and Gage, 2000) and SVZ (Doetsch et al., 1999).

However, it is now accepted that adult neurogenesis also happens in the CVOs (Bennett et al., 2009; Hourai and Miyata, 2013; Lin et al., 2015). The AP, as a CVO, is thus considered a newly discovered neurogenic niche in the adult mammalian brain. Because acute amylin injections affected several gene pathways and processes leading to the generation of new neurons, we hypothesized that chronic amylin administration would increase the constitutive neurogenesis in the rodent AP. In fact, our results demonstrated that sustained amylin infusion, not only decreased food intake, but significantly increased the number of newly proliferating cells in the adult AP (Figure 18, point 5) and drove the cell differentiation into a neuronal rather than glia lineage (Figure 18, point 6). These findings are consistent with previous evidence showing that amylin has the potential to protect from neuronal damages and to rescue impaired neurogenic functions (Adler et al., 2014; Trevaskis et al., 2010c).

However, the physiological relevance of the neurogenic effect of amylin in the AP is not known yet. The lack of a fully functional BBB makes the AP a "window of the brain", thus exposing it to all the potentially dangerous substances circulating in the blood, which may in turn damage the AP-neurons. Also, chronic amylin infusion might generate the necessity to activate more amylin-responsive neurons to avoid the instauration of amylin sensitivity. Therefore, the AP-ability of self-renewing could be a protective mechanism to maintain a functional signal regarding the energetic status of the body.

Still, many open questions remain to be answered. There is the need to understand which role these newly-formed neurons are engaged in and how they are integrated into the pre-existing neuronal circuits. Moreover, it would be fundamental to understand which mechanisms are involved in the amylin-mediated adult neurogenesis. Previous studies demonstrate that amylin signaling involves the activation of ERK1/2 cascade and that pERK AP-neurons partially overlap with CTR AP-neurons (Potes et al., 2010b). Moreover the number of ERK phosphorylated neurons is higher 10-15 minutes after amylin stimulus, exactly when the satiating effect is at its maximum and thus proving a direct role of amylin on AP-neurons (Lutz et al., 1995; Potes et al., 2012). Furthermore, in ERK knockdown mice neurogenesis was shown to be abnormal, suggesting that a functional ERK1/2 signaling is required for the generation of new neurons (Satoh et al., 2011). All together, these results imply that amylin might induce neurogenesis by activating ERK1/2 cascade in the AP. Further studies are still required to investigate this possibility. Blocking ERK

signaling in the AP or administer acute and chronic amylin in ERK knockout mice would contribute to a better understanding of the amylin's mechanism in adult neurogenesis. Moreover, to investigate the potential of amylin to induce neurogenesis in other brain areas where constitutive neurogenesis already occurs (*e.g;* SGZ and SVZ) would be of great interest to evaluate the neurogenic capacity of amylin.

In conclusion, the work presented in this thesis confirms the fundamental role of amylin in the control of feeding behavior and energy balance and presents a novel role of amylin in the process of adult neurogenesis.

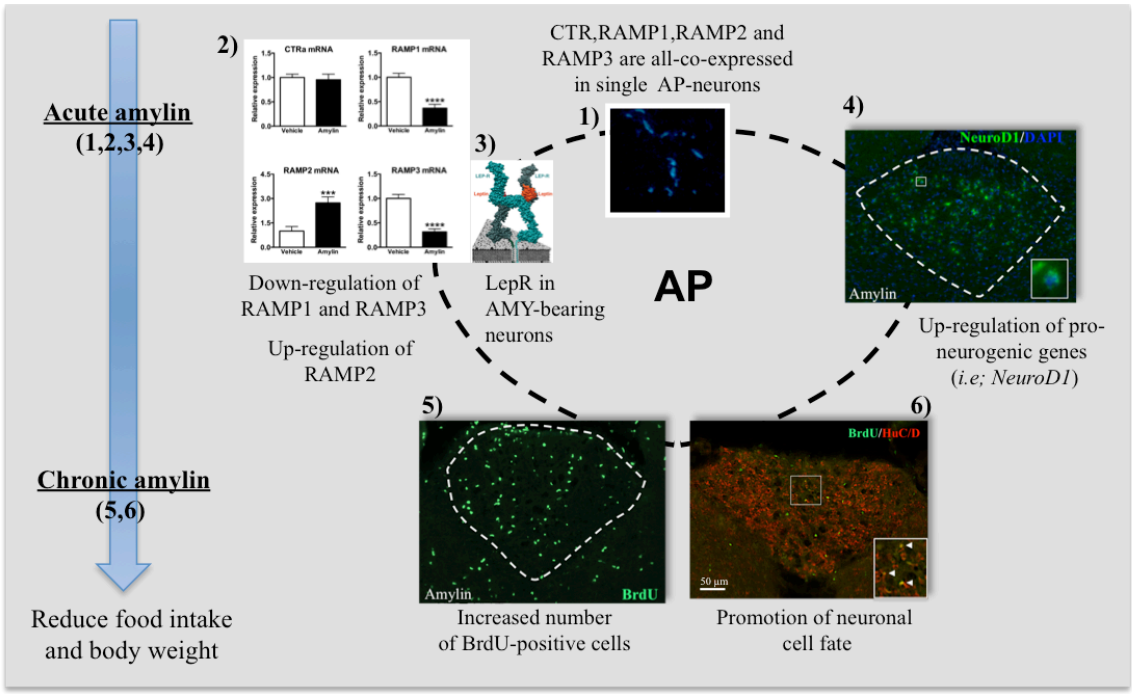


Figure 18: Effects of acute and chronic amylin administration on the rat AP.

Acute and chronic amylin administration decreased food intake and body weight in rodents. (1) All the necessary components to generate a functional AMY are co-expressed in single amylin-activated AP-neurons. (2) Acute amylin acts *via* a negative feedback regulatory mechanism to down-regulate RAMP1 and RAMP3, while RAMP2 and (3) LepRb were upregulated. Acute Amylin also regulated genes involved in neurogenic processes. (4) The transcriptional factor NeuroD1 was consistently up-regulated. Chronic amylin infusion (5) increased the number of newly proliferating BrdU-labeled cells in the AP, and (6) promoted the cell maturation into neurons rather than glia.

9. Abbreviations

AD	Alzheimer's disease
ADM	adrenomedullin
AMY	amylin receptor
Arc	nucleus arcuatus
AP	area postrema
BAT	brown adipose tissue
BBB	blood brain barrier
bHLH	beta helix-loop-helix
CLR	calcitonin-like receptor
CNS	central nervous system
CVO	circumventricular organ
CTRa	calcitonin-receptor isoform a
DG	dentate gyrus
DMH	dorso-medial hypothalamus
EE	energy expenditure
EphRs	ehprin receptors
ERK1/2	extracellular signal-regulated kinase 1 and 2
GO	gene ontology
HFD	high fat diet
JAK2/STAT3	janus kinase 2/signal transducer and activator of transcription 3
LBP	lateral parabrachial nucleus
LepRb	leptin receptor isoform b
LH	lateral hypothalamus
LHA	lateral hypothalamic area
ME	median eminence
NAc	nucleus accumbens
NeuroD	neuronal differentiation-1
NeuroD1	neuronal differentiation-1
NGS	next-generation sequencing
NSC	neural stem cell
NTS	nucleus tractus solitarious

OVLT	organum vasculosum of the lamina terminalis
PB	parabrachial nucleus
RAMP1	receptor-activity modifying protein-1
RAMP2	receptor-activity modifying protein-2
RAMP3	receptor-activity modifying protein-3
sCT	salmon-calcitonin
SGZ	subgranular zone
SFO	*subfornical organ*
SVZ	subventricular zone
T2D	type 2 diabetes
VMH	ventro medial hypothalamus
VTA	ventral tegmental area

10. References

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11. *Curriculum Vitae*

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Education

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PhD in Integrative Molecular Medicine Faculty of Science, University of Zurich
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10/2008 03/2011
International Master in Neuroscience, University of Trieste-International School for Advanced Studies (SISSA-ISAS), Trieste, Italy
Title: „Production of various forms of Alpha-Synuclein in the study of the etiological basis of Parkinson's disease „

10/2004 – 04/2008
Bachelor in Biology-Ecology, University of Parma, Parma, Italy
Title: „Sampling of industrial waste water in the light of the Environmental Law. Instruments and methods used by ARPA (Agenzia Regionale Protezione Ambiente) in Brescia„

09/1998 – 06/2004
Secondary School
Scientific High School (Liceo Scientifico) A. Calini, Brescia, Italy

Work Experience- *Special courses attended*

26-30/04 2010 (Trieste, Italy)
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LTK Module 5E: How to manage the breeding for an experiment.

Languages

Italian: Mother tongue
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Amylin receptor components and the leptin receptor are co-expressed in single rat area postrema neurons.
Liberini CG, Boyle CN, Cifani C, Venniro M, Hope BT and Lutz TA.
EJN 2015 Dec 22

The satiating hormone amylin induces neurogenesis in the area postrema of adult rats.

Liberini CG, Borner T, Boyle CN, and Lutz TA.

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